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Attorney's Docket Number: 18377-0012

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

U.S. Application No. (if known, see 37 CFR 1.5): 10/089130

International Application No.	International Filing Date	Priority Date Claimed
PCT/AU00/01184	22 September 2000	24 September 1999

Title of Invention
RECOGNITION OF DIFFERENCES IN CELL CYCLE STRUCTURE BETWEEN STEM AND DIFFERENTIATED CELLS

Applicant(s) for DO/EO/US
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Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), and (9) and (21) indicated below.
4. ☐ The US has been elected by the expiration of 19th month from the earliest claimed priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☒ is attached herewith (required only if not transmitted by the International Bureau).
   b. ☐ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ An English language translation of the International Application into English (35 U.S.C. 371(c)(2))
   a. ☐ is attached herewith.
   b. ☐ has been previously submitted under 35 US.C. 154(d)(4).
   c. ☒ translation not required as the application was filed in English.
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are attached herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 20. below concern document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☐ A FIRST preliminary amendment.
14. ☐ A SECOND or SUBSEQUENT preliminary amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 – 1.825.
18. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. ☐ Other items or information:

ATTACH CUSTOMER BAR CODE LABEL BELOW

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AO 700178.1

U.S. Application No. (if known, see 37 CFR 1.5) 10/089130	International Application No. PCT/AU00/01184	Attorney's Docket Number 18377-0012

21. ☒ The following fees are submitted: CALCULATIONS PTO USE ONLY

BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)):

Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO.. **$1040.00**

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$890.00**

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$740.00**

International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$710.00**

International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**

ENTER APPROPRIATE BASIC FEE AMOUNT =				$ 1,040.00	
Surcharge of $130.00 for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				$	
Claims	Number Filed	Number Extra	Rate		
Total claims	108 - 20 =	88	x **18.00**	$ 1,584.00	
Independent Claims	8 - 3 =	5	x **84.00**	$ 420.00	
Multiple Dependent Claims (if applicable)			+ **280.00**	$ 280.00	
TOTAL OF ABOVE CALCULATIONS =				$ 3,324.00	
☒ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by ½.			-	$ 1,662.00	
SUBTOTAL =				$ 1,662.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				$	
TOTAL NATIONAL FEE =				$ 1,662.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property			+	$	
TOTAL FEES ENCLOSED =				$ 1,662.00	
				Amount to be refunded:	$
				charged:	$

a. ☒ A check in the amount of **$1,662.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. 19-5029 in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment, to Deposit Account No. 19-5029. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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Recognition of differences in cell cycle structure between stem and differentiated cells

The present invention relates to methods for using molecules relating to the control of the cell cycle, and cell proliferation, to improve technology relating to pluripotent, multipotent and differentiated cells. More particularly, the present invention relates to methods for identifying pluripotent cells and partially differentiated cells, to methods for enhancing the maintenance and proliferation of pluripotent cells and partially differentiated cells, to methods for isolating new populations of pluripotent cells and partially differentiated cells, and facilitating their maintenance and proliferation *in vitro*, to methods for reprogramming of differentiated somatic cells so that the cells are converted to a less differentiated state, including to a state of pluripotency or multipotency, and to methods for selecting dedifferentiated cells, including those derived by reversion of differentiated or partially differentiated cells, in a mixed population of cells comprised of differentiated and dedifferentiated cells.

This invention also relates to methods for regulating the differentiation of cells, including pluripotent cells and multipotent cells, and to methods for prolonging the lifespan in vitro of pluripotent, multipotent or differentiated cells. Also within the scope of the present invention are cells, embryos and animals produced using the methods referred to above. In addition, uses of the cells, embryos and animals derived by these methods are within the scope of this invention.

In this patent application the term "pluripotent" refers to cells that can contribute substantially to all tissues of the developing embryo. "Multipotent" or "partially differentiated" refers to partially differentiated cells that are able to differentiate further into more than one terminally differentiated cell type. Such cells include, but are not limited to haematopoietic stem cells and neural stem cells.

"Maintenance of pluripotent cells" is to be understood as the

maintenance of such cells *in vitro* in an undifferentiated state. It may also include, but does not always include, the understanding that these cells are immortal.

Control of the cell division cycle and roles for cell cycle regulators in chromatin remodelling

The cell division cycle is normally composed of four distinct phases, which in typical somatic cells take 18-24 hours to complete. The S-phase represents the period when chromosomal DNA is duplicated, this is then followed by a gap phase (G2) where cells prepare to segregate chromosomes between daughter cells during M-phase. After completion of M-phase, cells enter a second gap phase, G1, which separates M- from S-phase. G1 is of special significance because it is here that a cell decides to continue dividing or withdraw from the cell cycle.

At the molecular level, the cell cycle is controlled by waves of cyclin-dependent protein kinase (Cdk) activities that are activated only at specific times and which drive the cell cycle transitions by phosphorylation of specific substrates. For activity, each Cdk catalytic subunit requires a cyclin regulatory subunit. Cdks acting at the G1 phase include Cdk2 which is regulated by cyclin E, and Cdk4 and Cdk6 which are regulated by cyclin D activities. Additional levels of control are provided by cyclin-dependent kinase inhibitors, such as p16.

Mitogenic signals, under normal circumstances, dictate whether a cell divides or arrests (Figure 1). The major step in mitogen-dependent control of cell proliferation comes at a point in G1, known as the Restriction Point (R-point), where cells commit to entering S-phase and to another round of cell division. At the molecular level, mitogen-dependent intracellular signalling pathways control this 'proliferative switch' by activation of cyclin-associated Cdk activities, which function primarily by phosphorylating the retinoblastoma tumour suppressor protein (pRb) and its family members, including p107 and

p130. This then allows for the dissociation of pRb from E2F transcription factors, resulting in derepression of target genes essential for the G1 to S transition. On the other hand, failure to activate cyclin-associated kinase activities will leave hypo-phosphorylated pRb associated with E2F, and exit from the cell cycle will follow (Figure 1). Significantly, loss of R-point control is an important step in tumorigenesis (Hall & Peters, 1996; Palermo & Peters, 1996). Another G1 Cdk activity, consisting of Cdk2 and cyclin E, collaborates with Cdk4,6-cyclin D to fully phosphorylate pRb and to promote the G1-S transition. Under some circumstances, elevated levels of cyclin E can relieve the requirement for cyclin D-associated Cdk activities, thus bypassing the R-point (Jiang et al, 1998) and cells lacking the pRb-pathway have a reduced requirement for Cdk4-cyclin D activities (Sherr & Roberts, 1995) and can pass from G1 through to S-phase using Cdk2 activity. Another Cdk2 activity, this time associated with cyclin A, is important for progression through S-phase in typical somatic cells (Pagano et al, 1992).

Cell cycle regulation clearly involves transcriptional control through pRb and its effects on E2F transcription factors. There is also now an emerging view that the molecules effecting cell cycle regulation are also linked to chromatin remodelling and differentiation. For example the transcriptional activities of pRb are associated with changes in chromatin structure (Brehm & Kouzarides, 1999). Furthermore histone acetyl transferases have a well established role in global control of gene expression and chromatin remodelling, with consequent effects on cell cycle regulation and differentiation (Kouzarides 1999). Changes in the activities of chromatin remodelling complexes have, in fact, been heavily implicated in the processes of cellular differentiation in a wide range of mammalian cell types (Kawasaki et al, 1998). In summary cell cycle regulation, and in particular cyclin-Cdk regulation, is associated with transcriptional control and changes in chromatin structure, and is likely to play an important role in global profiles of gene expression and differentiation.

Proliferation and differentiation during development

During mammalian development the differentiation of pluripotent cells and partially differentiated cells is coordinated closely with changes in proliferation.

In the mouse, development of the embryo in the first few days following implantation is a very dynamic period characterised by rapid cell proliferation and differentiation. At the time of implantation at 5.0 days post coitum (dpc) the embryo comprises a central ball of Inner Cell Mass (ICM) cells which are pluripotent (they can give rise to all cell types of the later embryo and adult). These are surrounded by the extraembryonic primitive endoderm and trophectoderm lineages. Around 5.0dpc the pluripotent cells, referred to from this time as epiblast, commence a period of rapid proliferation which accompanies transition from the ICM ball of cells, to a unicellular layer of pluripotent primitive ectoderm (Figure 2). The entire embryo arises from differentiation of the primitive ectoderm into the three germ layers ectoderm, endoderm and mesoderm during gastrulation which initiates at around 6.5dpc with the appearance of a structure called the primitive streak which forms at the posterior region of the embryo (Figure 2). Pluripotent cells migrate through the streak losing pluripotence and emerge as differentiated germ layer cells. From this time pluripotent cells in the embryo are restricted to the future germ cells.

Immediately following implantation the region of the embryo that will contribute to the adult, the primitive ectoderm, consists of around 30 cells, which divide once every 10-12 hours. This rate of cell division is marginally faster than that in the intestinal crypt, the most rapidly cycling cells in the adult. The embryo maintains this rapid rate of cell division in the primitive ectoderm cells for the next 48 hours. Just before gastrulation, the cell cycle of the primitive ectoderm decreases even further to around 6 hours (Figure 2). Indeed some propose the existence of a sub-population of cells within the primitive ectoderm with even faster cell cycle times of 3 to 4 hours. This is a

quite remarkable shortening of the cell cycle, especially when compared with the length of most somatic cell cycles, which range from 18-24 hours (Figure 2). Accelerated proliferation within the embryonic epiblast therefore precedes, or coincides with, the differentiation of primitive ectoderm and hence, loss of unlimited differentiation potential associated with pluripotency.

The very rapid expansion of the primitive ectoderm generates sufficient cell numbers to support formation of the three germ layers over a short developmental time-frame. This burst of cell proliferation is considered to be the force which drives gastrulation and can account for the transformation of a single layered embryo into the three germ layers, the definitive ectoderm, mesoderm and endoderm. Cell division in the newly formed mesoderm is not considered to play a significant role in expansion of this layer, nor the endoderm. Cell cycle length in the newly formed mesodermal cells has been measured at 12 hours and given that a complete mesodermal layer forms within 24 hours of the onset of gastrulation, most of the increase in this germ layer must be contributed from elsewhere, namely the primitive ectoderm (Hogan et al, 1994).

In the post-gastrulation embryo it appears that all cells have cycles of >12 hours. It is therefore apparent that in the early postimplantation embryo the rate of proliferation firstly increases dramatically just prior to and during gastrulation, but then slows after the cells lose pluripotency in the post-gastrulation embryo, and at some time they differentiate.

In the mammal the close association between differentiation and alterations in proliferation continues beyond embryonic development, and extends throughout adult life.

Multipotent stem cell populations play a critical role in mammalian development and in normal renewal of differentiated cells. Many differentiated cells are not replaced by proliferation of existing differentiated cells, but by the

differentiation of partially differentiated (precursor) cells called multipotent stem cells. Multipotent stem cells are not terminally differentiated themselves, but are competent to differentiate into one or more terminally differentiated cell types (multipotent differentiation potential). For example the stem cell for haemopoiesis can differentiate into at least 9 different kinds of blood cells. The rates of renewal and differentiation of stem cells are coupled to control the rate of differentiated cell production and prevent depletion of the stem cell population. Multipotent stem cells have a capacity for renewal which, if not infinite, extends beyond the lifetime of the animal.

The changes in the rate of cellular proliferation during embryogenesis and the continued close association between differentiation and changes in proliferative rates during later development have been described previously (Hogan et al, 1994), but the cell cycle structure and molecular mechanisms underlying these changes have not been rigorously evaluated.

The availability of murine pluripotent cells in vitro has led to the development of powerful model systems for investigating mechanisms of early development. In particular pluripotent cells provide an opportunity to investigate the molecular events responsible for the close association between pluripotency and cell cycle characteristics, and the link between differentiation and changes in regulation of the cell cycle.

Murine pluripotent cells can be isolated from the preimplantation embryo and maintained in vitro as ES cells. ES cells retain pluripotence indefinitely and display the properties of stem cells, including competency to differentiate into all cell types, and the ability for indefinite self-renewal. Early primitive ectoderm-like (EPL) cells are also pluripotent stem cells. They differ in some properties to ES cells, and have the capacity to revert to ES cells in vitro. They can be derived from ES cells or other types of pluripotent cells, and are the in vitro equivalent of primitive ectoderm cells of postimplantation embryos. As such, EPL cells can also be established in vitro from cells isolated from the

primitive ectoderm of postimplantation embryos. The properties of EPL cells, factors required for their maintenance and proliferation in vitro, and their ability to differentiate uniformly in vitro to form essentially homogeneous populations of partially differentiated and differentiated cell types are described fully in PCT/AU99/00265, to applicants, the entire disclosure of which is incorporated herein by reference. Cells of the primordial gonad, primordial germ cells (PGCs), also retain pluripotency during embryonic development, and can be isolated and cultured in vitro as embryonic gonadal (EG) cells. Embryonic carcinoma (EC) cells may also be pluripotent.

While pluripotent cells and partially differentiated cells have long been recognised as ideally suited to a range of applications, in practice technical barriers have generally restricted their use in the prior art. Specific opportunities and shortcomings of stem cell technology include:

Genetic Modification of Livestock

The potential for genetic modification of livestock species for agricultural, medical and commercial application is enormous. Proof of concept has been achieved by application of traditional pronuclear injection techniques for the expression of therapeutic proteins in ruminant milk (Janne et al, 1998). However, the genetic modification of animals for applications such as xenotransplantation (Platt & Lin, 1998), has been severely limited by inherent shortcomings in pronuclear injection technology. Firstly, integration of the injected DNA is inefficient and random, leading to difficulties in achieving appropriate transgene expression. Secondly, modification of endogenous genes is possible only in exceptional circumstances.

ES cell and nuclear transfer technologies have the potential to overcome these deficiencies:

Relatively robust technologies have been developed for the creation of mice that are genetically modified in a predetermined manner (Bradley et al,

1992). Methodologies based on homologous recombination are used for efficient and precise genetic alteration of endogenous genes in ES cells, including alteration of individual nucleotides. Genetically modified ES cells are then used as a vector to transmit the genetic modification through the germline of chimaeric animals to all cells of F1 offspring.

The ability to genetically alter commercially valuable species in a precise way has been limited by the inability to generate stable, proliferating pluripotent cell lines from non-rodent species. A novel approach for improved isolation and maintenance of pluripotent cells in vitro from a wide range of species, that were previously refractory to isolation by conventional approaches would be a significant advance in the art. Widespread in vitro availability of robust pluripotent cells from commercially useful species would thereby provide an opportunity for their precise genetic manipulation and use in agricultural, commercial and medical applications.

The creation of Dolly the cloned sheep (Wilmut et al, 1997) by nuclear transfer identified a second potential route to precise genetic modification of mammals. It is envisaged that genetic modification will be carried out in primary somatic cell cultures by homologous recombination technologies developed for use in mouse ES cells. The nuclei from genetically modified cells would be used as karyoplast for nuclear transfer to create genetically altered animals. A problem with this technology is that the efficiency of nuclear transfer is low; only a small proportion of nuclear transfer embryos develop to live animals. This inefficiency is due largely to problems with reprogramming of the somatic nucleus to a pluripotent state after transfer to the recipient oocyte.

Novel Human Therapies

A second area in which stem cell technology is expected to have commercial impact is in the development of human therapeutics for gene therapy and cell-based therapy (Smith, 1998; Rathjen et al, 1998). These technologies will be used for treatment of diseases in which cell replacement is

likely to be of value (ie Parkinson's, chronic viral infection), for correction of genetic defects (gene therapy), and for delivery of protein pharmaceuticals. Alternative strategies are based on the use of somatic multipotent stem cells and embryonic stem cells. The attraction of somatic stem cells for gene therapy is that, being immortal, administration of genetically altered stem cells should provide long term cures for genetic disease.

Significant problems have been encountered in the realisation of this technology. In many cases, identification and culture of stem cells for target tissues has not been achieved. Furthermore, even when cell populations can be enriched, for example in the case of haemopoietic stem cells, these have proven refractory to genetic manipulation. In particular the failure of cultured stem cells to proliferate clonally in culture prevents the use of homologous recombination-based techniques for modification of endogenous genes.

Human application of ES cell technologies provides a route of great promise for the development of novel human therapies (Smith, 1998; Rathjen et al, 1998). Briefly, human ES cells would be differentiated in vitro to an appropriate cell type for transplantation. The ES cells could also be genetically modified using the homologous recombination based technologies prior to controlled differentiation, and used as a cell-based therapy for genetic diseases. This technology is creating enormous interest because the nature of both the genetic modification and the transplanted cell type would be unrestricted. This circumvents important limitations to current gene therapy protocols. A barrier to this technology is the requirement for transplanted cells to evade immune rejection. One solution to this might be creation of a generic human `donor' ES cell line, modified to escape immune surveillance. A preferred approach is the development of techniques, which allow the isolation of `individual-specific' pluripotent cells. This can potentially be achieved via the use of nuclear transfer technology where pluripotent cells are isolated from viable embryos generated by injecting somatic cell karyoplasts into an oocyte cytoplast. This approach raises important ethical considerations. An alternative

approach that circumvents these ethical problems is to generate individual-specific pluripotent cells directly from somatic cells, without formation of a viable embryo, by dedifferentiating somatic cells in vitro (Smith, 1998; Rathjen et al, 1998). A method of dedifferentiation remains a deficiency in the prior art, however.

There are further applications of pluripotent cells or multipotent cells, and in particular autologous pluripotent cells or multipotent cells, identified and/or derived by manipulation of the cell cycle regulatory molecules according to the present invention. For example these cells may be used after differentiation in vitro, for the regulated delivery of drugs. Such cells may be manipulated in vitro to express genes necessary for production and secretion of required drugs, and transplanted into appropriate tissues. For example, insulin producing cells could be generated for the controlled delivery of insulin, for the treatment of diabetes. In addition these pluripotent cells and multipotent cells may be used in organ development and regeneration, and limb and appendage growth and replacement. They also may be used as diagnostics, and in the preparation of pharmaceuticals.

It is clear that there have been major difficulties in the successful isolation, maintenance in vitro, genetic manipulation and germ-line transmission of pluripotent cells from species other than mouse. These difficulties have severely restricted the application of these technologies for commercial, medical and agricultural benefit.

There have also been difficulties that have restricted the successful reprogramming of differentiated cells so that they revert to a pluripotent, or less differentiated state. In particular there have been difficulties in trapping cells in a pluripotent or partially differentiated state following spontaneous dedifferentiation, or dedifferentiation occurring as a result of an environment or factors that promote dedifferentiation. Similarly approaches for the selection of pluripotent cells or partially differentiated cells from cell populations comprised

of pluripotent cells, partially differentiated cells and differentiated cells have been limited. Nuclear transfer has been one of the approaches suggested in the prior art to achieve reprogramming of differentiated somatic cells. However nuclear transfer approaches have been inefficient due at least in part, to difficulties in reprogramming. Furthermore there are major ethical problems with the use of human oocytes in reprogramming human somatic cells.

There have also been problems in controlling the differentiation of pluripotent cells along defined differentiation pathways.

There have also been difficulties associated with primary somatic cells in vitro. In particular it has been difficult to maintain primary cells in culture for prolonged periods, due to biological mechanisms that limit the number of proliferation rounds that such cells can undergo. The consequence of this limitation, termed the Hayflich limit, is that the ability to genetically modify primary or untransformed cells in vitro has been restricted. This limitation has restricted the utility of these cells for commercial, agricultural or medical benefit.

It is accordingly an object of the present invention to overcome or at least alleviate one or more of the difficulties or deficiencies associated with the prior art.

Applicants have discovered that the unusual mode of cell cycle regulation seen in pluripotent cells is an intrinsic feature of the pluripotent state and that maintenance of this state is dependent, in part at least, on the behaviour and properties of molecules that are involved in cell cycle regulation/cell proliferation. Accordingly, these molecules may be used to identify, isolate and maintain populations of pluripotent cells in vitro, that are otherwise unmanageable. This may also facilitate the identification and isolation of cells that have dedifferentiated back to a less differentiated state.

Identification of pluripotent cells and multipotent cells

Accordingly, in a first aspect of the present invention there is provided a method for identifying pluripotent cells on the basis of their cell cycle dynamics and expression and/or activity of cell cycle regulatory molecules.

Specifically, there is provided a method for identifying pluripotent or pluripotent-related cells which method includes

analysing the cell population for pluripotent cell cycle characteristics including one or more of

pluripotent-specific cell cycle structure;

pluripotent-specific expression and/or activity of cell cycle regulatory molecules; and

phosphorylation status of a tumour-suppressor protein(s).

In a preferred aspect the identification method may include measuring expression of cell cycle regulatory molecules, including a cyclin(s), a cyclin-dependent protein kinase (Cdk), a Cdk inhibitor, upstream regulators of said molecules or biochemical targets thereof. The method may also include determining the phosphorylation state of a tumour suppressor protein such as pRb and other family members such as p107 and p130.

It was known in the prior art that ES cells proliferate rapidly, with a cell cycle of about 10-12 hours in vitro. However little was known previously about the cell cycle structure, and the molecular events regulating the cell cycle in pluripotent cells.

The applicant has surprisingly found that ES cells and other pluripotent cells including EPL cells, and pluripotent cells in the developing embryo have a different cell cycle structure compared to differentiated cells. Pluripotent cells spend the majority of time (~65%) in S phase, and short proportions of the cell cycle in G1 (~15%) and G2/M (~20%) phases. In the case of G2 and M-

phases, it is likely that pluripotent cells lack a G2 phase, as the total G2/M period can be accounted for by the time required for M-phase (mitosis and cytokinesis) alone. Upon differentiation, the cell cycle of pluripotent cells is remodelled and adopts a profile typical of that seen in other somatic cells (ie. full gap phases become obvious). For example in definitive mesoderm, the percentage of time spent in S-phase is reduced to 15-20% and the G1 phase expands to 60%. Applicants conclude that the cell cycle profile of pluripotent cells in vitro and in vivo is significantly and identifiably different from differentiated cells both from the embryo and in cultured primary and transformed cells. The restricted lengths of G1 and G2 phases suggest that regulatory controls such as checkpoints might be lost in stem cells, making them less responsive to mitogenic signals.

Applicants have also found that molecular events regulating the cell cycle in pluripotent cells are different to those in differentiated somatic cells. Firstly pluripotent cells express high levels of cyclin E. Furthermore cyclin E-kinases are constitutively active at levels more than 50 times those seen in rapidly dividing primary somatic cells. Similarly pluripotent cells may express high levels of cyclin A associated activities.

Applicants have also found that the expression profiles of the INK family of Cdk inhibitors are distinctly different in pluripotent cells compared to differentiated cells, and that expression patterns of INK-Cdk inhibitors are associated with pluripotency. Notably the Cdk inhibitor p16, which inhibits activities of cyclin D-associated Cdks, cyclin D/Cdk4 and/or cyclin D/Cdk6, but does not inhibit cyclin E Cdk activity or cyclin A activity or is present in very low levels, is not present in pluripotent cells. Similarly the expression of the Cdk inhibitors p21 and p27 are substantially reduced or eliminated in pluripotent cells.

Applicant has also found that the tumour suppressor Retinoblastoma protein (pRb) is maintained in an inactive state by phosphorylation in

pluripotent cells. Accordingly pRb is unable to interact with the E2F transcription factor, and E2F-activated genes are expressed throughout the cell cycle.

Accordingly in a preferred embodiment of this aspect of the present invention, the identification method may include measuring one or more of the following:

(a) cell cycle structure (pluripotent cells have a rapid cell cycle, with short gap phases).

(b) cyclin E (a high level of constitutive expression activity of cyclin E being characteristic of pluripotent cells);

(c) cyclin A (pluripotent cells typically contain elevated cyclin A levels)

(d) phosphorylation status of pRb (phosphorylation and therefore inactivity of pRb being a characteristic of pluripotent cells);

(e) INK, Cip or Kip family of Cdk inhibitors, for example:

(i) p16 and/or p21 and/or p27 (lack of expression or very low levels of expression is characteristic of pluripotent cells)

In a preferred embodiment, the pluripotent-related cells may include multipotent cells (such as haemopoietic stem cells and neural stem cells). The multipotent cells may be derived by partial differentiation of pluripotent cells and which are capable of differentiating further into a number of different cell types, which may have all or some of the above cell cycle features.

Accordingly, pluripotent cells may be identified by the presence of one or more of the following characteristics:

a pluripotent-specific cell cycle exhibiting a rapid cycle with short gap phases;

elevated constitutive expression and/or activity of cyclin E;

elevated constitutive expression and/or activity of cyclin A;

substantial reduction in, or absence of, expression of Cdk inhibitor p16,

substantial reduction in, or absence of, expression of Cdk inhibitors p21 and/or p27; and

presence of an inactive (phosphorylated) tumour suppressor protein.

Cells with properties similar to the cell cycle characteristics found in pluripotent cells are identified as pluripotent cells.

Preferably the pluripotent or pluripotent-related cells are identified by the presence of one or more of the following characteristics:

a pluripotent-specific cell cycle exhibiting a rapid cycle with short gap phases;

elevated constitutive expression and/or activity of cyclin E;

elevated constitutive expression and/or activity of cyclin A;

pluripotent-specific expression of Cdk inhibitors; and

presence of a phosphorylated tumour suppressor protein.

In a further embodiment of this aspect of the present invention, the pluripotent-related cells may include multipotent cells (such as haemopoietic stem cells and neural stem cells). The multipotent cells may be derived by partial differentiation of pluripotent cells and which are capable of differentiating further into a number of different cell types, which may have all or some of the above cell cycle features.

In a further aspect of the present invention, there is provided

a method of identifying differentiating cells, which method includes analysing the cell population for differentiating cell cycle characteristics including

differentiation-specific cell cycle structure;

differentiation-specific expression and/or

activity of cell cycle regulatory molecules, including

the presence of active tumour suppressor proteins.

In one embodiment, the identification method may be utilised to monitor the onset of differentiation.

The cell cycle structure, expression and activity of cell cycle regulatory molecules are altered significantly during differentiation. The alterations may include one or more of the following:

In relation to the cell cycle, the gap phases of the cell cycle become more prominent, and the rapidity of the cell cycle slows.

Differentiating cells are increasingly dependent on tumour suppressor proteins, such as pRb and other family members for regulation of the cell cycle.

cyclin E-is significantly downregulated in differentiating cells, through reduced gene expression, and reduced protein stability.

cyclin A-associated activities are also reduced.

Cdk 2 activities become cell cycle regulated.

The activities of other cell cycle regulatory molecules, such as the INK Cdk inhibitor p16 and/or other INK family members are upregulated.

The activities of other Cdk inhibitors p21 and/or p27 are upregulated.

Accordingly differentiating cells may be identified by the presence of

one or more of the following characteristics:

a differentiation-specific cell cycle exhibiting a relatively slow cycle with prominent gap phases; and/or

tumour suppressor protein dependency;

reduced constitutive expression activity of cyclin E and/or cyclin A;

Cdk2 activities being cell cycle regulated.

Increased expression of the Cdk inhibitors p16, and/or p21 and/or p27.

Maintenance and proliferation of pluripotent and multipotent cells.

In a further aspect, the present invention provides a method of regulating the mitotic and/or physiological activities, and differentiation potential of a pluripotent or multipotent cell, which method includes

manipulating the expressing and/or activity of a cell cycle regulatory molecule including a regulatory molecule selected from one or more of the groups consisting of a cyclin, a cyclin-dependent protein kinase (Cdk), a Cdk inhibitor, upstream regulators thereof or biochemical targets thereof and/or tumour suppressor protein, and molecules displaying similar activities, in a pluripotent or multipotent cell.

The method of regulation described above may be applied to facilitate maintenance and/or promote proliferation to enhance pluripotent or multipotent *in vitro*, such that some or all of the features of the cell cycle of pluripotent cells are enforced.

Applicants' difficulties in maintaining and proliferating pluripotent cells *in vitro* may be overcome by enforcing the features of the cell cycle of pluripotent cells. Enforcement of these characteristics may be achieved using several alternative approaches in combination or separately.

In one embodiment, maintenance and proliferation of pluripotent cells in

vitro can be achieved by enforcement of cyclin E activity. In another embodiment it can be achieved by enforcement of cyclin A activity.

Maintenance and proliferation of pluripotent cells *in vitro* may also be achieved by enforcing Cdk2 expression, (the cyclin-dependent kinase activity normally regulated by cyclin E and cyclin A), so that Cdk2 activity is constitutive and independent of cyclin E or cyclin A regulation. Methods include upregulation of Cdc25, an activator of Cdk2 activity, and/or down regulation of wee 1-like activity, which down-regulates Cdk2 activity. Manipulation of activities associated with cyclin D may also be used for maintenance and proliferation of pluripotent cells. These manipulations may allow cyclin D activities to substitute for cyclin E and A activities. Manipulations include constitutive upregulation of cyclin D activity, or the cyclin dependent kinases Cdk4 and/or Cdk6 (the Cdks normally regulated by cyclin D). Up regulation of these Cdks may also be achieved by Cdc25.

In a preferred embodiment, manipulation of upstream regulators of cell cycle regulatory molecules may also be used to achieve maintenance and proliferation of pluripotent cells. For example the proto-oncogenes *myc* (Amati et al, 1998) and *ras* are known upstream regulators of cyclin E activity and cyclin A activity. Traditionally research and commercial applications have focussed on reducing the activity of such proto-oncogenes, for applications that include treatment of cancers. It is a novel approach to upregulate these proto-oncogenes for applications such as proliferation and maintenance of pluripotent cells.

The lifespan of pluripotent cells *in vitro* may also be prolonged by manipulating the activities and expression of tumour suppressor molecules, such as pRb, and related activities, p107 and p130. In general cell cycle entry may be promoted by hyperphosphorylation and inactivation of tumour suppressor proteins such as pRb, leading to activation of the E2F family of transcription factors. Inactivation of pRb may be achieved by

hyperphosphorylation, or by other approaches that include antisense technology, or gene inactivation. Similarly prolonged lifespan, proliferation and continued maintenance of differentiation status may be achieved by constitutive expression or activity of E2F transcription factors. For example, constitutive E2F activity would occur when E2F is manipulated so that it no longer interacts with nor inhibited by pRb or other tumour suppressors.

The pluripotent cells may be of any suitable type and may be *in vitro* or *in vivo*. Preferably, the pluripotent cells are selected from one or more of the group consisting of epiblast cells, ES cells, EPL cells (as described in International Patent application PCT/AU99/0265), primordial germ cells (PGCs), or embryonic carcinoma (EC) cells.

Similarly the multipotent cells may be of any suitable type and may be in vitro or in vivo. They may be any partially differentiated cell type, including such cells as haematopoietic stem cells and neural stem cells.

Where the cell cycle regulatory molecule includes a cyclin, the cyclin may be of any suitable type. Preferably the cyclin is cyclin D, cyclin E, cyclin A or a molecule exhibiting similar activity (e.g. virally encoded cyclins that are not inhibited by Cdk inhibitors), or a functionally active fragment or analogue thereof. The cyclin-dependent protein kinase may be of any suitable type and includes biochemical activities with similar properties. Preferably the Cdk is Cdk4, Cdk6, or Cdk2, or a molecule exhibiting similar activity, or a functionally active fragment or analogue thereof.

The upstream regulatory pathways may be components of signalling pathways that, in some cases, are known to modulate aspects of cell behaviour such as, but not limited to, cell proliferation, the cell cycle and differentiation status. This would include and is not limited to molecules such as myc family members, including c-myc, l-myc and n-myc, Ras, Raf, MAP kinase, Rho and other signalling pathways. Ultimately the activity of these

molecules would serve to provide and maintain intracellular activities required for stem cell maintenance separate to or, in conjunction with, the cell cycle functions described.

The activities of said cell cycle regulatory molecules may be manipulated by any suitable technique. Such techniques include but are not limited to manipulation of expression of said proteins, including manipulation of gene expression for example by transformation with expression constructs, antisense technology or fusion protein technology where the fusion protein includes a transduction domain linked to the cell cycle regulatory molecule, or any other protein delivery system such as electroporation or lipofection. Such techniques are well known to those skilled in the art and are described in, for example, Sambrook et al (1989), the entire disclosure of which is incorporated herein by reference.

In a preferred form the manipulated cell cycle regulatory molecule is an upstream regulator of cyclin E and/or cyclin A, such as c-myc and other members of the myc family. In this preferred form c-myc activity is upregulated by ectopic gene expression, or most preferably by protein transduction, using a fusion protein that includes a transduction domain linked to the cell cycle regulatory molecule, or any other protein delivery system such as electroporation or lipofection.

Methods for extending the lifespan in vitro, of primary cells and untransformed cells

There have also been difficulties associated with the short lifespan of primary somatic cells and untransformed cells in vitro. In particular there are biological mechanisms that limit the number of proliferation rounds that such cells can undergo. This difficulty is termed the Hayflich limit. One consequence of this difficulty is that the ability to genetically modify primary or untransformed cells in vitro is restricted, limiting their potential applications in a range of technologies, including nuclear transfer.

The lifespan in vitro of primary and untransformed cells may be prolonged by manipulation of cell cycle regulatory activities.

Accordingly, in accordance with this aspect of the present invention, there is provided a method of regulating the cell cycle of primary and untransformed cells, which method includes

manipulating the expression and/or activity of a cell cycle regulatory molecule, such that the proliferation and maintenance of differentiated cells are reduced, wherein the regulatory molecule is selected from one or more of the groups consisting of a cyclin, a cyclin-dependent protein kinase (Cdk), a Cdk inhibitor, upstream regulators thereof or biochemical targets thereof and/or tumour suppressor protein, and molecules displaying similar activities, in a primary or untransformed cell.

In an alternative or supplementary embodiment, the lifespan of such cells *in vitro* may be prolonged by manipulating the activities and expression of tumour suppressor molecules, such as pRb, and related activities, p107 and p130. In general, cell cycle entry may be promoted by hyperphosphorylation and inactivation of tumour suppressor proteins such as pRb, leading to activation of the E2F family of transcription factors. Inactivation of pRb may be achieved by hyperphosphorylation, or by other approaches that include antisense technology, or gene inactivation. Similarly prolonged lifespan, proliferation and continued maintenance may be achieved by constitutive expression or activity of E2F transcription factors. For example, constitutive E2F activity would occur when E2F is manipulated so that it no longer interacts with nor inhibited by pRb or other tumour suppressors.

Methods for selecting pluripotent cells

In another aspect of the present invention there is provided a method of selecting for pluripotent cells from a mixed cell population including pluripotent cells, and differentiated cells which method includes

manipulating the activity of a cell cycle regulatory molecule, such that the proliferation and maintenance of differentiated cells are reduced, wherein the regulatory molecule is selected from one or more of the groups consisting of a cyclin, a cyclin-dependent protein kinase (Cdk), a Cdk inhibitor, upstream regulators thereof or biochemical targets thereof and/or tumour suppressor protein, and molecules displaying similar activities, in a pluripotent or multipotent cell.

The differentiated cells may be already present in the cell population, or derived by differentiation of pluripotent cells. Elimination of differentiated cells from the population would in itself improve overall pluripotent cell stability, since differentiated cells are capable of promoting differentiation of pluripotent cells. Hence this method confers an additional advantage in enhancing pluripotent cell stability.

The preferential inhibition of differentiated cell maintenance and proliferation without inhibiting pluripotent cell maintenance and proliferation may be achieved by manipulation of any of the cell cycle regulatory molecules including cyclins, cyclin-dependent protein kinases, and Cdk inhibitors, or their upstream regulators or down stream targets. Upstream regulators include the protooncogenes from the *myc* family, which upregulate cyclin E-associated activities and cyclin A-associated activity. Other upstream signalling pathways such as Ras, Raf, MAP kinase, or Rho may also be used.

In a preferred embodiment preferential inhibition of differentiated cell maintenance and proliferation is achieved by manipulating Cdk inhibitor activity. Pluripotent cells would remain insensitive to the manipulated Cdk inhibitor activity, whereas progress of differentiated cells through the cell cycle is preferentially inhibited.

In a particularly preferred embodiment, the activity of the Cdk inhibitor p16 is manipulated. Constitutive p16 activity in pluripotent cells does not inhibit

cyclin E/Cdk2 activity or cyclin A/Cdk2 activity so that pluripotent cells are insensitive to the Cdk inhibitor p16. Hence p16 does not inhibit proliferation of pluripotent cells while cells are in an undifferentiated state. However cyclin D/Cdk4 and/or cyclin D/Cdk6 activity is inhibited by p16 Cdk inhibitor activity in differentiated cells, preventing differentiated cell proliferation and initiating differentiated cell death. In this embodiment, enforced expression of p16 in a cell population comprised of pluripotent and differentiated cells, leads to preferential survival of the pluripotent cells. Other members of the INK family of Cdk inhibitors, or other molecules with overlapping activities may also be used.

In a further preferred embodiment constitutive p16 activity or constitutive activity of other INK family members with similar activity may be achieved by ectopic gene expression or any other means. Most preferably Cdk inhibitor activity is achieved by protein transduction, using a fusion protein that includes a transduction domain linked to p16, or any other protein delivery system such as electroporation or lipofection.

In a similar manner, constitutive Cdk inhibitor activity such as p16, or other INK family members with similar activity may also be used to maintain and select for multipotent cells, such as haematopoietic or neural stem cells.

In other embodiments of this approach, any activity or molecule that regulates the cell cycle, and/or confers a susceptibility to differentiated cells, and an insensitivity to pluripotent cells may be used. For example the proto-oncogene *myc* is an upstream regulator of the cell cycle promoting proliferation in pluripotent cells, but induces apoptosis in some differentiated cells. Hence ectopic *myc* expression may also be used to select for cells reverting to a less differentiated state.

In yet another embodiment, preferential inhibition of differentiated cell maintenance and proliferation could be used in combination with approaches

to promote pluripotent cell maintenance and proliferation. For example a Cdk inhibitor such as p16 may be used to inhibit differentiated cell maintenance and proliferation, in combination with cyclin E and/or cyclin A, or upstream regulators of cyclin E- and/or cyclin A-associated activities such as the myc family of proto-oncogenes to promote maintenance and proliferation of those pluripotent cells selected on the basis of their insensitivity to p16 or other INK family members with similar activity.

In another form, manipulation of cell cycle regulatory molecules can be used to preferentially select for partially differentiated cells from a mixed cell population comprised of partially differentiated cells and differentiated cells.

Use of this approach to trap cells that spontaneously revert to a less differentiated state.

More particularly, these methods can be exploited to obtain cells that have reverted from a differentiated state to a less differentiated state, in a manner that is independent of nuclear transfer. Reversion to a less differentiated state may occur spontaneously. In differentiated cell populations, including multipotent cell populations, it is probable that spontaneous reversion to a less differentiated state occurs at a low frequency, or rarely. Reverted cells may be trapped in their less differentiated state, and selected for by the previously described methods.

In one embodiment enforced Cdk inhibitor expression, and in particular p16 expression (p16 or other INK family members with similar activity is the preferred Cdk inhibitor) is achieved in a differentiated cell population by techniques that include ectopic gene expression or any other means. Most preferably Cdk inhibitor activity is achieved by protein transduction, using a fusion protein that includes a transduction domain linked to p16, or any other protein delivery system such as electroporation or lipofection. . Differentiated cells, which rely on cyclin D-associated activities for proliferation, are sensitive to p16 activity, and fail to proliferate, and undergo cell death. Rare individual

cells within these populations that revert to a pluripotent state or less differentiated state are trapped in their reverted pluripotent or less differentiated state by ectopic p16 activity.

Reprogrammed cells may then be maintained in their pluripotent or less differentiated state by this approach or in combination with other approaches described in this application. For example preferential selection of pluripotent cells may be used in combination with methods to promote the maintenance and proliferation of selected cells as described above.

Use of this approach to select and capture reprogrammed cells derived by environmental reversion.

Several reports support the emerging view that partially differentiated cells, or multipotent stem cells are more amenable to reprogramming than previously thought. For example bone marrow derived cells have been found in several non-haematopoietic tissues following transplantation, including vascular endothelial cells (Shi et al, 1998) and brain (Eglitis & Mezey, 1997).

With current art it is not possible to direct reprogramming to derive cells of a desired cell type. Neither is it possible to identify cells in transitional stages of reprogramming, as only terminally differentiated cells formed by reprogramming can be identified.

Non spontaneous reversion may be initiated by placing multipotent or differentiated cells in an environment in vitro that promotes programming. The methods described in this application for the selection of pluripotent cells or multipotent cells can be adapted to trap and temporarily lock cells in the transitional stages of reprogramming. One likely possibility is that environmental signals are able to direct reprogramming through a transitional pluripotent state, and subsequent differentiation through all germ line lineages. In this approach the signals that promote reprogramming are derived from pluripotent cells, or from cells in early differentiation, as occurs in embryoid

bodies. Hence reprogramming is initiated by incorporating cells destined for reprogramming in a pluripotent cell population or into embryoid bodies. Cells responding to these inductive environmental signals reach a transitional pluripotent or multipotent state. Cells passing through these transitional phases can be trapped, and used to prepare essentially homogeneous populations of partially differentiated or differentiated cells, using the methods described in PCT/AU99/00265.

The methods described in the present invention for the selection of pluripotent cells or multipotent cells may be used to trap cells in the transitional phases of reprogramming.

In one embodiment, it is postulated that reprogramming directed by environmental signals occurs by transition through a pluripotent state, and subsequent differention through all germ line lineages. Such cells in a transitional pluripotent state may be trapped, and maintained in a pluripotent state by the methods described in this application.

Capture of cells in a transitional pluripotent state and their maintenance may be achieved by manipulation of any of the cell cycle regulatory molecules including cyclins, cyclin-dependent protein kinases, and Cdk inhibitors, or their upstream regulators or down stream targets. Upstream regulators include the protooncogenes from the myc family, which upregulate cyclin E-associated activities and cyclin A-associated activities. Other upstream signalling pathways such as Ras, Raf, MAP kinase, or Rho may also be used.

A preferred embodiment includes utilisation of Cdk inhibitors as described above.

A particularly preferred embodiment utilises p16, or other molecules, including other INK family members with overlapping activities, more preferably utilising p16.

The Cdk inhibitors may also be used in combination with other methods for maintenance and proliferation of pluripotent cells, such as manipulation of cyclin E, or upstream regulators such as myc family etc.

These methods may also be used to capture and maintain cells in a transitional multipotent state, formed by reprogramming in response to an inductive environment.

Methods for reprogramming of differentiated or partially differentiated cells, to a less differentiated state, including to a state of pluripotency

Manipulation of the activities of cell cycle regulatory molecules also provides approaches for the dedifferentiation of differentiated cells.

Accordingly in this aspect of the present invention, there is provided a method for reprogramming of differentiated or partially differentiated cells to a less differentiated state which method includes

manipulating the expression and/or activity of a cell cycle regulatory molecule, including a regulatory molecule selected from one or more of the groups consisting of a cyclin, a cyclin-dependent protein kinase (Cdk), a Cdk inhibitor, upstream regulators thereof or biochemical targets thereof and/or tumour suppressor protein, and molecules displaying similar activities, in said differentiated or partially differentiated cells.

For example, cell cycle regulatory molecules or their upstream regulators or downstream targets are manipulated in differentiated cells, so that their cell cycle properties assume at least some of the cell cycle properties of pluripotent or multipotent cells.

Dedifferentiation may be achieved by manipulation of any of the cell cycle regulatory molecules including cyclins, cyclin-dependent protein kinases,

and Cdk inhibitors, or their upstream regulators or down stream targets. Upstream regulators include the protooncogenes from the myc family, which upregulate cyclin E-associated activities. Other upstream signalling pathways such as Ras, Raf, MAP kinase, or Rho may also be used.

Examples include cyclin(s), particularly upregulation of cyclin E, cyclin A; cyclin-dependent protein kinases, particularly Cdk2; Cdk inhibitors; and upstream regulators of cyclin E- and/or cyclin A-associated activities, and other upstream pathways; particularly myc.

Dedifferentiation may be achieved by manipulation alone, or in combination with other procedures that promote dedifferentiation, such as nuclear transfer.

In the prior art, the efficiency of nuclear transfer is low, and only a small proportion of nuclear transfer embryos develop to live animals. This inefficiency is due largely to problems with reprogramming of the somatic nucleus to a pluripotent state after transfer to the recipient oocyte. Reprogramming efficiency can be improved by manipulating cell cycle properties of donor somatic cells so that they more closely resemble cell cycle features of a pluripotent cell, prior to nuclear transfer.

In a preferred embodiment, manipulation of cell cycle regulation in differentiated cells or partially differentiated cells in this way, may prime cells so that they are more readily dedifferentiated by nuclear transfer. Pluripotent cells may be formed from manipulated cells by fusion with enucleated oocytes, or cytoplasts derived from pluripotent cells.

Methods for regulating differentiation of pluripotent cells by manipulation of cell cycle.

Applicant has discovered that the unusual structure of pluripotent cell cycles and their unique mode of molecular regulation are intimately related to their stem cell state. The cell cycle structure, expression and activity of cell cycle regulatory molecules are altered significantly during differentiation, and these changes in pluripotent cells in vitro and in vivo are causally or mechanistically linked with differentiation.

During differentiation of pluripotent cells the gap phases of the cell cycle become more prominent, and the rapidity of the cell cycle slows. Tumour suppressor proteins, such as pRb and other family members are increasingly involved in the regulation of the cell cycle as the pluripotency is lost, and cells differentiate. In such cells the activities of some cell cycle regulatory molecules may be downregulated by comparison with their activities in pluripotent cells. For example cyclin E-associated activities are significantly downregulated in differentiating cells, and cyclin A-associated activities are also reduced. The activities of other cell cycle regulatory molecules, such as the INK Cdk inhibitor p16 may be upregulated, and other Cdk inhibitors such as p21 and/or p27 may also be upregulated.

Accordingly there is provided a method of regulating the differentiation of pluripotent or multipotent cells by manipulating the expression and/or activity of a cell cycle regulatory molecule, including a regulatory molecule selected from one or more of the groups consisting of a cyclin, a cyclin-dependent protein kinase (Cdk), a Cdk inhibitor, upstream regulators thereof or biochemical targets thereof and/or tumour suppressor protein, and molecules displaying similar activities, in said cells.

Preferably, the differentiation of the pluripotent or multipotent cells is regulated by one or more of the following manipulations:

increasing the activity of the tumour suppressor protein;

reducing cyclin E and/or cyclin A-associated activities;

the activity of Cdk2 becomes cell cycle regulated; or

upregulating the activities and/or expression of CdK inhibitors.

Methods include:

increasing the role of tumour suppressor proteins, such as pRb and other family members in regulation of the cell cycle.

cyclin E-downregulation by methods that include reduced gene expression (eg antisense technology) and/or reduced protein stability.

reducing cyclin A-associated activities, e.g. by methods that include reduced gene expression (eg antisense technology) and/or reduced protein stability.

Cdk 2 activities become cell cycle regulated.

Upregulate the expression and/or activities of Cdk inhibitors such as p16, p21 and p27.

The present invention will now be more fully described with reference to the accompanying figures and examples. It should be understood, however, that the description following is illustrative only and should not be taken in any way as a restriction on the generality of the invention described above.

In the Figures:

Figure 1 illustrates regulation of the cell cycle.

Figure 2 illustrates remodelling of the cell cycle during embryonic development.

Figure 3 illustrates flow cytometry analysis of pluripotent embryonic epiblast cells isolated from day 6.5dpc embryos.

Figure 4 illustrates comparison of cell cycle profiles between 6.5dpc embryonic epiblast, ES, EPL and NIH 3T3 fibroblasts.

The general cell cycle structure and cell cycle length are indicated. Note the relatively short cell cycle length and lack of fully formed gap phases in pluripotent ES, EPL and epiblast cells.

Figure 5 illustrates cell cycle remodeling during differentiation of cells in embryoid bodies.

ES cells or EPL cells grown as EPL embryoid bodies in the absence of LIF were fixed and stained with propidium iodide. EPL bodies grown in the absence of LIF were harvested to evaluate changes in cell cycle structure associated with differentiation. Note the relative increase in the proportion of G1 cells and a decrease in the proportion of S-phase cells as cells differentiate.

Figure 6 illustrates E2F target genes are not cell cycle regulated in ES cells.

Top panel: ES cells were synchronized by the nocodaole-aphidicolin block –release protocol. Following release from the G1/S aphidicolin block, cells were harvested, RNA was prepared and resolved on 1% formaldehyde agarose gels, blotted onto a nitrocellulose membrane and probed with a P^{32}-labelled cyclinE, RRMP-2 or mGAP cDNA fragment. The timing of entry into S-phase for synchronous ES cell populations is indicated. Lower panel:

Synchronous populations of NIH3T3 cells were collected at varying times after refeeding serum starved cells with 10% FCS. RNA was prepared and levels of mGAP and cyclinE mRNA evaluated as described above. Note the cyclical changes in cyclinE mRNA levels during successive cell cycles in NIH3T3 cells compared to the lack of cell cycle oscillation in pluripotent ES cells.

Figure 7 illustrates E2F complexes in pluripotent cells are predominantly free of pRb family members.

Whole cell extracts from ES, EPL, MEF and NIH3T3 cells were incubated with a P^{32}-labelled consensus binding site for the E2F transcription factor. Complexes were resolved on non-denaturing polyacrylamide gels. The faster migrating complexes represent DNA-E2F/Dp1/2 complexes (Dp proteins bind with E2Fs and form heterodimeric transcription factor complexes). Slower migrating complexes represent those that have recruited pRb family members and is indicative that the pRb/family member protein is in a hypophosphorylated state. Note that in pluripotent cells such as ES and EPL, E2F is predominantly in the free (active) form and is uncomplexed with pRb family proteins.

Figure 8 illustrates E2F4 is the major E2F activity in ES cells.

Electrophoretic mobility-shift assays were performed as described in Figure 7. The major E2F activity in ES cells is shown to be E2F4 by supershifting the E2F complex with an antibody that specifically recognizes

E2F4. The major pRb-like activity is shown to be p107, by using the super-shift assay, with an anti-p107 antibody.

Figure 9 illustrates Cyclin E and cyclin B associated activities in ES cells and NIH3T3 cells.

Whole cell extracts from ES or NIH 3T3 cells were prepared from unsynchronized (untreated), +aphidicolin treated (G1/S block) or nocodazole blocked (G2/M) cells. 50• g total protein from each extract was used in immunoprecipitation assays with anti-cyclinE or anti-cyclinB antibody. Kinase activities in the immunoprecipitates were evaluated using recombinant GST-pRb or purified histone H1 as an in vitro substrate.

Figure 10 illustrates levels of cylin E and cyclin A are not cell cycle regulated in ES cells.

ES cells were synchronized and released into S-phase. At 90 minute intervals, samples of cells were collected, whole cell extracts were prepared and levels of Cdk4, cyclinE, cyclinB, cyclinA, Cdk2 and Cdc2 detected by Western blotting. Note that in ES cells, cyclinB levels vary in a cell-cycle dependent manner (ie levels peak late in G2/M-phase). CyclinE and cyclin A levels do not vary throughout the cell cycle however.

Figure 11 illustrates Cyclin E and cyclin A activities and mRNA pluripotent cells and during differentiation in embryoid bodies.

ES cells were converted into EPL cells, followed by growth of EPL cells as embryoid bodies. Bodies were grown in the absence of LIF for the number of days indicated (0-5days) and the extent and rate of differentiation determined using molecular markers by Northern blot hybridization (Oct4, marker of pluripotency; brachyury, marker for nascent mesoderm; FGF5, marker for primitive ectoderm, mGAP, loading control). During the time-course of the assay, levels of cyclin E, cyclin A and Cdk2 were determined and compared to that in early passage MEFs. Cyclin E kinase activity was determined in cell extracts prepared at different times before and during differentiation. Note the precocious activity of cyclinE/A-kinase activity in pluripotent cells compared to MEFs.

Figure 12 illustrates p21 and p27 mRNA and protein levels in differentiating EPL embryoid bodies.

p21 and p27 mRNA were detected by Northern blot hybridization in cells undergoing differentiation as EPL embryoid bodies. Levels of p27 protein were monitored using a specific antibody that recognizes p27 (see Material and methods) in a parallel experiment.

Figure 13 illustrates c-myc expressing ES cell lines have a reduced requirement for LIF.

LIF titration assays were performed as described in Materials and methods. After 6 days growth in the designated concentration of LIF, colonies

were stained for alkaline phosphatase activity. The data shows the % colonies staining positive for alkaline phosphatase. Each data point is the average of assays performed in duplicate.

Figure 14 illustrates ectopic expression of c-myc severely reduces the ability of pluripotent cells to differentiate.

ES cells were grown into EPL embryoid bodies and their ability to differentiate monitored using molecular markers (Oct4, pluripotency; brachyury, nascent mesoderm). RNA was prepared from embryoid bodies at different times after withdrawal of LIF (1-5 days). RNA was subject to agarose gel electrophoresis and individual RNAs detected by Northern blot. Note that c-myc expressing cells do not switch off Oct4 (and hence, retain pluripotency) and only turn on the differentiation marker (brachyury) at low levels. Data for two c-myc and one control cell line is shown. Levels of ribosomal RNA are shown in the right hand panel.

Figure 15 illustrates Tat-p16 fusion protein selectively inhibits the growth of NIH3T3 fibroblasts but not ES cells.

Varying concentrations of TAT-p16 or TAT-p16mut (0-150• g/ml) were added to sub-confluent cultures of NIH3T3 or ES cells. Cells were counted at time points after addition of TAT fusion protein. Each experiment was performed in duplicate and data points represent average values.

EXAMPLE 1: The cell cycle profile of pluripotent cells *in vitro* and

epiblast cells *in vivo* are similar, and are strikingly different to the profile in differentiated cells.

To demonstrate that the mode of cell cycle regulation is fundamentally different in pluripotent and differentiated cells, we performed flow cytometry analysis (based on DNA content) on purified populations of epiblast cells derived from mouse embryos and on pluripotent cells derived from in vitro culture.

Materials and Methods

Isolation of pluripotent cells from 6.5dpc embryos:

Isolation of pluripotent cells from 6.5dpc embryos were by dissection from time-mated Swiss mice, Reichardt's membrane and the extra-embryonic part removed, before the visceral endoderm peeled away from the epiblast by pipetting the embryo up and down with a narrow bore pipette. Each epiblast at this stage of development consists of between 750-1000 cells making it necessary to pool approximately 10 embryos to generate sufficient cell numbers for flow cytometry analysis. After generating a single cell suspension, cells were then fixed and stained with propidium iodide (described in Dunphy 1997) for flow cytometry analysis on the basis of cellular DNA content (Figure 3). This analysis allows the relative proportion of time spent in G1, S-phase and G2/M to be determined.

Cell culture in vitro:

Mouse embryonic stem (ES) cells were cultured in the absence of feeders on tissue-culture grade plastic-ware (Falcon) pre-treated with 0.2% gelatin-PBS for a minimum of 30 minutes. Cells were cultured in Dulbecco's Modified Eagles Medium (GIBCO BRL) pH7.4 containing high glucose. To generate the final DMEM used for cell culture this was supplemented with 10% foetal calf serum (FCS; Commonwealth Serum Laboratories), 40mg/ml gentamycin, 1 mM L-glutamine and 0.1 mM • -mercaptoethanol (• -ME). DMEM was supplemented with 1000 units of LIF under 10% CO_2 in a humidified incubator where appropriate. Routine tissue culture was performed as described by Smith (1991).

LIF was routinely produced from COS-1 (ATCC CRL-1650) cells transfected with a mouse LIF expression vector plasmid, pDR10, as described by Smith (1991). COS-1 cells were transfected by electroporation using a Bio Rad Gene Pulsar at 270 Volts and a capacitance of 250 • FD. Transfected cells were plated at $7X10^4$ cells/cm^2 in DMEM, pH 7.4, containing high glucose and supplemented with 10% FCS, 40 • g/ml gentamycin and 1mM L-glutamine. Medium was collected and assayed for LIF expression as described by Smith (1991). Alternatively, medium was supplemented with 1000 units of recombinant LIF (ESGRO, AMRAD).

E14, CGR8, E14TG2a ES cells (Hooper et al 1987), MBL5 (Pease et al 1990) and D3 (Doetschman et al., 1985) ES cells were cultured in DMEM supplemented with LIF as described above. Embryonal carcinoma cell lines were F9 and P19 (ATCC CRL 1825). Mouse embryo fibroblasts were isolated

from day 12.5 Swiss mouse embryos as described in Hogan et al 1994. Other cell lines including NIH3T3 fibroblasts (ATCC CRL1658), Balb/c 3T3 clone A31 (ATCC CCL 163) were grown in DMEM supplemented with 10% FCS.

Hep G2 cells (Knowles et al., 1980; ATCC HB-8065) were maintained in culture in DMEM and passaged at confluence. To condition medium (MEDII) Hep G2 cells were seeded into DMEM at a density of 5 X 10^4 cells/cm^2. Medium was collected after 4-5 days, sterilized by filtration through a 0.22 • m membrane and supplemented with 0.1 mM • -ME before use. MEDII was stored at 4°C for 1-2 weeks or at -20°C for up to 6 months with apparent loss of activity.

EPL cells were formed and maintained in media containing 50% MEDII conditioned medium in DMEM with or without the addition of LIF. EPL formation was apparent with the addition of between 10 and 80% MEDII, with optimal culture conditions at 50% MEDII.

Pluripotent EPL cells were formed from ES cells and maintained as follows;

Adherent cultures: ES cells were seeded at a density of 1 X 10^5 cells/cm^2 onto tissue culture grade plastic-ware (Falcon) pre-treated with 0.2% gelatin/PBS for a minimum of 30 minutes in DMEM containing 50% MEDII as described above. EPL cells were maintained in 50% MEDII using routine tissue culture techniques (as described by Smith, 1991).

Suspension aggregates: ES cells were seeded at a density of 1 $X10^5$ cells/cm^2 in suspension culture in bacterial petri dishes in DMEM containing 50% MEDII as described above. The resulting EPL cell aggregates were split 1:2 after 2 days and seeded into fresh DMEM containing 50% MEDII. Growth of embryoid bodies (EPL or ES) were described in Rathjen et al 1999.

Flow cytometry analysis: Cell cycles profiles were determined by propidium iodide staining of fixed cells as described in Dunphy (1997).

Western blot analysis: Antibodies used for Western blot and immunoprecipitation analysis were as follows. cyclin E (Santa Cruz sc-481 or sc-198), p16 (Santa Cruz sc-1207), pRb (Becton Dickinson 14001A), p107 (Santa Cruz sc-318), E2F-4 (Santa Cruz sc-866), Cdk2 (Santa Cruz sc-163), p27 (Santa Cruz sc-528), p21 (Santa Cruz sc-397). HRP-conjugated secondary antibodies raised against total rabbit or mouse immunoglobulins were from Dako Corporation (P217 and P0260, respectively). Detection of proteins in Western blot analysis was by the Pierce ECL kit (#34080).

Results

Our results reveal some striking features of epiblast, ES and EPL cell cycles relative to cell cycles of differentiated somatic cells. First, it is apparent that pluripotent cells, including those of the epiblast, spend the majority of their time in S-phase (~65%) and short proportions of the cell cycle in G1 (~15%) and G2/M (~20%) phases (Figures 3,4). In the case of G2 and M-phase cells

(which can't be discriminated on the basis of DNA content), we suspect these cells lack a G2 phase as the total G2/M period can be accounted for by the time required for M-phase (mitosis and cytokinesis) alone. These findings are consistent across all of the pluripotent cells tested including, pluripotent cells in the embryonic epiblast, ES cell lines (D3, E14, CGR8, E14TG2a, MBL5), EC cells lines (F9, P19) and EPL cells (Rathjen et al 1999). This strikingly contrasts the cell cycle structure normally associated with differentiated cells; for example in NIH 3T3, Balb/c 3T3 where the % time spent in G1 is approximately 65%. This is accompanied by a significantly longer generation time (~22-24 hours).

Clearly, an aspect of the pluripotent state, of cells in the embryo and of cells grown in culture, is that they have a unique cell cycle structure that accompanies their rapid proliferative potential (Hogan et al 1994). As pluripotent EPL cells are allowed to differentiate into mesoderm (following formation of EPL embryoid bodies in the absence of LIF from EPL cells; Rathjen et al 1999), their cell cycle profile is restructured so that accompanying their increased cell cycle length, there is an acquisition of full gap-phases (Figure 5).

The potential consequences of shortened G1 and G2/M phases in pluripotent cells, could be decreased cell cycle length and loss of regulatory controls such as checkpoints. Molecules known to modulate the length of the gap phases and of cell cycle length when ectopically expressed include cyclin E (Resnitzky et al 1994) and c-myc (Kam et al 1989), respectively. The

molecular mechanisms underlying changes in cell cycle structure and rates of cell division in pluripotent cells and multipotent cells have not been addressed previously and neither has the relevance of this to the establishment and maintenance of the pluripotent state.

EXAMPLE 2: Regulation of the cell cycle in pluripotent cells.

Material and Methods

All cells and tissue culture techniques were as described in Example 1 unless otherwise stated.

Northern Blot Analysis and in vitro Cdk assays:

Cytoplasmic RNA preparation and Northern blot analysis were by standard methods (Sambrook et al., 1989). In vitro kinase assays were performed as described in Dunphy (1997).

Synchronization of ES cells

A synchronization procedure, taking into account the unusual nature of the pluripotent cell cycle structure, was developed and optimized. ES cells were first plated as single cell suspensions at 5 X 10^5 cells/ml onto gelatin-coated petri dishes (see Example 1). After 12-14 hours culture in DMEM plus LIF, the microtubule depolymerizing agent nocodazole (Sigma), was added to a final concentration of 45ng/ml. After 8 hours, cells were washed three times

in DMEM (37°C) and then incubated for a further 5-8 hours in DMEM plus LIF supplemented with 5• g/ml aphidicolin (Sigma), which blocked the cell population at the G1/S border. Cells were released from the G1/S cell cycle block by washing three times in DMEM supplemented with LIF (warmed to 37°C). During each wash cycle, cells were kept under standard culture conditions for five minutes between media changes. Cell cycle arrest, synchrony and cell cycle transit was evaluated by flow cytometry (see Example 1) to evaluate cell DNA content (data not shown). After release from cell cycle blocks, cell samples were taken and used for cell cycle analysis (for example; protein extracts, RNA extractions, flow cytometry analysis).

Results

pRb activity in pluripotent cell cycles

The retinoblastoma tumour suppressor protein, pRb, and its family members (p107, p130) are key regulators of the G1-S transition and are crucial for processes that normally control cell proliferation. Their activity is controlled by the activity of cell cycle regulated Cdk activities which impose a distinct pattern of cell cycle regulated phosophorylation on pRb-family members (Dyson 1998). Underphosphorylated pRb is generally accepted to be the active form, capable of interacting with cellular targets such as the E2F family of transcription factors (Dyson 1998). The hyperphosphorylated form is the inactive species, and one measure of this is the loss of pRb's ability to associate with E2F transcription factors. Loss of pRb function in tumour cells is commonly associated with deregulated E2F activity and has several

implications for the general aspects of cell cycle regulation including a reduced requirement for cyclin D-Cdk4,6 activities and consequently, an acquired insensitivity to the Cdk inhibitor p16 (see Jiang et al 1998 and references therein).

To establish the role of these key regulators in the control of pluripotent cells, a biochemical analysis of pRb function was performed. We have evaluated pRb phosphorylation status by Western blot analysis of ES, EC and EPL cell extracts prepared from a nocodazole-aphidicolin synchronization protocol developed in our laboratory. Similar parallel experiments were performed for differentiated cells derived from pluripotent cell populations. pRb protein levels do not change significantly throughout the cell cycle in synchronous pluripotent cells, but suprisingly is found exclusively in a slow migrating form (data not shown), indicating that it is maintained in a phosphorylated state throughout the cell cycle. No hypophosphorylated (active) pRb was ever detected. This suggests that pRb may be inactive and unable to interact with E2F. In such circumstances, upstream regulators of the pRb pathway such as Cdk4,6-cyclin D complexes and the Cdk inhibitor p16 are not required. These molecules are frequently inactive in tumour cells lacking pRb activity (Jiang et al 1988).

The ability of pRb to inhibit E2F transcription factors was determined by measuring the expression of E2F-target genes that are normally activated during the G1-S period of the cell cycle (Figure 6). Northern analysis was conducted on RNA isolated from synchronised ES cells, and probed for cyclin

E and RRMP-2 mRNA (RRMP-2 refers to a ribonucleotide reductase subunit). Both these genes are normally transcribed by E2F transcription factors and are cell cycle regulated in differentiated somatic cells. mGAP mRNA levels remain constant throughout the cell cycle, and was used as a control to show relative RNA loading. Results shown in Figure 6 indicate that these genes are active throughout the cell cycle and not subject to any cell cycle-regulated repression/activation. Hence, E2F target genes are constitutively active, and pRb (and p107, p130) regulation of E2F activity does not exist in ES cells. This contrasts the general situation in primary and established cell lines other than pluripotent cells, where E2F target genes are tightly cell cycle-regulated through Cdk regulation of pRb-family proteins. Hence, this shows a fundamental difference in the cell cycle regulatory pathways that operate in pluripotent stem cells.

The ability of pRb to interact with E2F transcription factors was tested using gel mobility shift analysis (Figure 7). Our work identifies E2F-4 as the major E2F DNA binding activity in ES cell nuclear extracts; it is this E2F activity that is shifted by an anti-E2F antibody in gel shift mobility assays (Figure 8). Remarkably, the vast majority of E2F activity in pluripotent cells was in the 'free' state, uncomplexed with pRb family proteins (Figure 7). No E2F-pRb complexes were detected by band shift analysis using supershifting anti-pRb antibodies to probe the composition of protein-DNA complexes (data not shown). Instead, a small fraction of E2F-4 is associated with p107, (another member of the pRb tumour suppressor family) although the majority of E2F-4

band-shift activity remains in the free/active form (Figure 7 and 8). This small amount of p107-bound E2F4 however, may be derived from the small fraction of differentiated cells in the pluripotent population. Similarly, no recruitment of p130 (a third member of the pRb tumour suppressor family) into E2F complexes has so far been observed in these experiments. We conclude from this data that E2F target genes are unlikely to be controlled by pRb or pRb-related factors, including p130 and p107, in a manner that has been established for E2F target genes in other cell types. The presence of mainly free (non pRb-complexed E2F) indicates that pRb family members are biochemically inactive through the activity of such molecules as Cdks. The consequence of this is that E2F target genes and the basic R-point pathway is non-functional. This is supported by our observations that pRb is never detected in the hypo-phosphorylated state, there is an absence of appreciable interaction between E2F transcription factors and pRb family members and finally, and E2F target genes are not cell cycle regulated. These observations show that pRb activity is absent in pluripotent cells. We believe that this, in part, could explain why the G1 phase of pluripotent cells is truncated relative to differentiated cell types and points towards an unusual mode of cell cycle regulation that could explain the unusual cell cycle structure described in Example 1.

Cdk2-cyclin e as a constitutively active regulator of pluripotent cells

To learn more about proliferative control in pluripotent cells, we have

investigated Cdk activities associated with Cdk2. The activity of Cdk2-cyclinE activity was evaluated in asynchronous (untreated), G2/M (nocodazole) or G1/S (aphidicolin) blocked cells. This experiment demonstrates an unusual mode of Cdk regulation in pluripotent ES and EPL cells because cyclin E-Cdk2 is active at unusually high levels (see direct comparison between pluripotent cells and early passage MEFs, with equal protein input in Figure 11) and moreover, is promiscuously active throughout the cell cycle (Figure 9). Under normal circumstances cyclin E-associated Cdk activities are tightly cell cycle regulated, peaking in G1/early S-phase. In pluripotent cells however, cyclin E-kinase activity is still active in a nocodazole arrest (G2/M block) which is normally associated with inactive cyclin E-kinases in differentiated cells. The mitotic cyclin B-associated kinase activity behaves as expected however, and is more active in G2/M cells than at other points in the cell cycle. This is consistent with the situation in differentiated cells (such as NIH 3T3 fibroblasts). Hence, the pluripotent state is associated with an unusual pattern of activity for some Cdk activities (cyclin E,cyclin A/Cdk2), but not all (Cdk1-cyclin B). Similar results were obtained in F9 and P19 pluripotent embryonal carcinoma cells and pluripotent EPL cells (data not shown), indicating that this unusual mode of Cdk regulation is a general property of pluripotent cells. Levels of cyclin E-Cdk2 pRb kinase activity are of comparable activity in G2/M cells and G1 cells and are significantly higher in magnitude than that found in MEFs, NIH 3T3s and other cell lines tested (Figure 9 and data not shown). After adjustment of total protein input, the amount of Cdk2 activity in pluripotent ES, EC and EPL cells was determined to be 20-50X higher than in

low passage mouse embryo fibroblasts (data not shown). The overall characteristics of Cdk2 regulation in pluripotent cells deviates significantly from the normal pattern of cyclin E-Cdk2 activity in differentiated somatic cells, where cyclin E-Cdk activity increases during G1 and collapses during S-phase, as a result of tight cell cycle regulatory mechanisms. Promiscuous Cdk2-cyclin A activity was also seen- ie absence of cell cycle regulation and elevated levels relative to primary and immortalized cell lines (see Figure 11 and data not shown).

In support of the continued cyclin E-Cdk2 activity throughout the cell cycle, cyclin E protein was shown to be present throughout the cell cycle in synchronised cells by Western analysis (Figure 10), at elevated levels. This contrasts the situation in other cell types where cyclin E levels are strictly cell cycle regulated due to transcriptional and post-transcriptional mechanisms (Sherr 1994). Mitotic cyclins however, such as cyclin B, exhibit cell-cycle dependency (Figure 10) as is typically seen in differentiated, non-pluripotent cells. This lack of cell cycle regulated control of cyclin E and cyclin A at the protein level is multifactorial and can be explained in part at least by, (i) the lack of cell cycle regulation of the cyclin E/cyclin A transcription (the cyclin E and cyclin A genes are E2F target genes) and, (ii) enhanced stability of the cyclin E/cyclin A proteins in pluripotent cells (data not shown).

In the early embryo, our data derived by immunohistochemistry shows that prior to gastrulation, cyclin E staining is intense in all cells of the epiblast (data not shown). This is consistent with the observation in ES cells that cyclin

E levels are invariant throughout the cell cycle. This contrasts the staining pattern in the surrounding visceral endoderm layer (data not shown) where general staining is less intense and only detected in a fraction of cells (this is probably coincident with G1 and S-phase cells). The precocious activities of Cdk2-cyclin E and Cdk2-cyclin A most likely underpin the constitutively phosphorylated state of pRb in pluripotent stem cells, hence explaining pRbs biochemical inactivity and constitutive activity of E2F target genes.

Additional work has shown that precocious cyclin E-Cdk2 activity is a special feature of the pluripotent state. As pluripotent ES and EPL cells are allowed to differentiate (following withdrawal of LIF), cylin E-Cdk2 activity was monitored in relation to well established differentiation markers for pluripotency, nascent mesoderm and primitive ectoderm: Oct4, brachyury and FGF-5, respectively (Figure 11). Our data shows that as pluripotent cells are allowed to differentiate (withdrawal of LIF), several key events occur. (1) cell cycle structure gets remodelled such that the gap phases become longer (see Figure 5), (2) cell cycle length becomes longer as a consequence of (1), (3) Cdk2 activities become severely down-regulated as cells lose pluripotency (Figure 11), (4) this is associated with down-regulation of cyclin E protein levels (Cdk2 protein remains constant, Figure 11) and (5) as part of the differentiation program, Cdk2-cyclin E and Cdk2-cyclin A activities become cell cycle regulated (data not shown). Loss of precocious cyclin E-Cdk2 and cyclin A-Cdk2 activities and establishment of their cell cycle regulation are mechanistically linked to the loss of pluripotency (see Figure 11).

The apparent unrestrained activity of Cdk2 kinases that persists in pluripotent cells throughout the cell cycle, occurs in the absence of two Cdk inhibitory molecules, p21 and p27 (Figure 12), known to modulate Cdk2 activity (Sherr and Roberts 1995). Besides being Cdk inhibitors for Cdk2 kinase complexes, p21 and p27 are established assembly factors for the Cdk4,6-cyclin D complex (Cheng et al 1999). These latter complexes are principally involved in control of the R-point by phosphorylating pRb and it's family members. In the absence of p21 and p27, little or no active Cdk4-cyclin D assembly activity would be expected, implying that these activities do not play a major role in the control of pluripotent cell proliferation. Upon cellular differentiation (after loss of pluripotency, for example) however, when p21 and p27 first become present, it would be expected that Cdk4,6-cyclin D activities would become crucial. Hence the requirement for Cdk4,6-cyclinD activities occurs as cells lose pluripotency and as Cdk2 activities become cell cycle regulated.

The presence of pRb in a biochemically inactive, hyper-phosophorylated state, suggests from other prescedents that pluripotent cells lack a functional R-point. Because these cells lack functional pRb, they should exhibit a reduced requirement for Cdk4,6 kinase activities (see Jiang et al 1998 and references therein). This situation can be satisfied if Cdk2 activities are elevated, as in the case of some tumour cell lines. Our data indicate that pluripotent cells also will also have a reduced requirement for Cdk4,6 activities as they also have elevated Cdk2 activities and lack functional pRb. The

absence of pRb activity is likely to be through a mechanism where precocious Cdk2-cyclin E/A holds it in a constitutively phosphorytlated state, independent of Cdk4,6 activities.

EXAMPLE 3: Identification of pluripotent and other stem cells

The successful isolation, maintenance in vitro, genetic manipulation and germ-line transmission of pluripotent cells from species other than mouse has generally been difficult and largely unsuccessful to date. One of the difficulties in obtaining pluripotent cells from other species has been the inability to demonstrate simply and rapidly, that putative pluripotent cells are pluripotent. Generally Oct-4 expression has been identified as a reliable marker for pluripotency. Alkaline phosphatase has also been used, but its absolute reliability as a pluripotent cell marker is questionable since a number of differentiated cells also express AP. Recently some novel markers that distinguish between different types of pluripotent cells were identified (PCT/AU99/00265). The markers L17 and Psc1 are expressed in ES cells and are down regulated in EPL cells. Conversely K7 is upregulated in EPL cells compared to ES cells.

We have found that the expression and_activity of cell cycle markers can be determined rapidly in vitro, and this data can be used alone, or in combination with other markers not directly involved with cell cycle regulation, to establish the pluripotent state or otherwise of putative pluripotent cell populations. In particular the type of cell cycle regulatory activities present,

cell cycle regulatory molecules expressed, and the phosphorylation state of pRb can be used as markers for pluripotency.

The following criteria can be used to identify pluripotent cells.

- Elevated levels and constitutive activity of cyclin E-Cdk2 activities, and cyclin E expression.

- Elevated levels and constitutive activity of cyclin A-Cdk2 activities and cyclin A expression.

- Inactive/constitutively phosphorylated pRb and/or other pRb tumour suppressor family members, such as p107 and p130.

Substantial reduction in, or absence of, the INK Cdk inhibitor p16.

Substantial reduction in, or absence of, the Cdk inhibitors p21 and p27.

Many differentiated cells in the mammal are not replaced by proliferation of existing differentiated cells, but by the proliferation of precursor cells (multipotent stem cells). Cellular decisions taken by stem cells are exquisitely regulated by external cues, which link stem cell behavior to the requirements of the organism. Rates of renewal and differentiation are coupled to prevent depletion of the stem cell population and control the rate of differentiated cell production, while the pathway of stem cell differentiation can be varied in response to environmental stimuli.

These cells combine a number of properties that are shared with pluripotent cells, and distinguish them from terminally differentiated somatic

cells.

They are competent to differentiate into one or more terminally differentiated cell types. For example the haematopoietic stem cells can differentiate into at least 9 different kinds of blood cell.

They are immortal, a property shared only by transformed cells.

They have a capacity for renewal which, if not infinite, extends beyond the lifetime of the animal.

Cell cycle markers and associated regulatory molecules, characteristic of pluripotent cells are also likely to be useful in identifying other stem cell populations. Although the cell cycles of ES cells are unusually short, this may not be a feature of other stem cell populations driven by cyclin E and for example having modified pRb-family member function. For example, haematopoietic stem cells can proliferate slowly, but at the molecular level, cell cycle regulation may be similar to ES cells.

In summary the criteria described above to identify pluripotent cells may also be used to identify multipotent cells, which may have some or all of the cell cycle activities and expression properties listed above.

Example 4: Maintenance and proliferation of pluripotent cells *in vitro* by manipulation of C-Myc gene expression

A major problem in obtaining pluripotent cells from species other than

mouse has been the inability to proliferate and maintain pluripotent cells in vitro. Manipulation of cyclin activity or activities that can enforce high cyclin-Cdk activities in putative pluripotent cells, offers one approach to overcome these difficulties. These approaches would mimic/reproduce essential characteristics of the stem cell-state.

For example constitutive expression of cyclin E from a transgene expression construct would force continuing rounds of cell proliferation by maintaining pRb in an inactive/phosphorylated state, and would promote constitutive transcription of E2F target genes. It is also likely that such cell cycle regulatory molecules are associated with chromatin remodelling and maintenance of pluripotent status (Brehm & Kouzarides, 1999; Kouzarides 1999). Alternatively, maintaining high Cdk activity through the established properties of upstream regulators such as c-myc (Amati et al 1998) is another approach to stabilize pluripotent cells in vitro. Another approach would be to enforce the elevated activity of down-stream effector molecules associated with these pathways.

Differentiation of pluripotent cells is associated with a change in cell cycle structure, changes in the rate of cell proliferation, establishment of cell cycle regulated Cdk activities and down regulation in the absolute activities of some Cdks. Maintenance of Cdk activities in a constitutive state would serve to maintain stem cells, prevent them from differentiating and to facilitate their propagation in culture for extended periods.

Materials and Methods

c-myc expression construct:

A construct suitable for the stable expression of human c-myc was constructed by inserting a Eco RI fragment, from the plasmid pSR• MSVtkNEO (Sawyers et al 1992) that contains the entire c-myc coding sequence, into the Eco RI site of the expression vector, pEF-IRES-puro (Hobbs et al 1998). This construct, pc-Myc.puro, utilizes the EF1• promoter and drives the transcription of the c-myc open reading frame in addition to the puroR gene by way of an internal ribosome entry site (IRES).

Establishment of stable c-myc expressing cells lines:

10• g of Ase I- digested pc-Myc.puro was electroporated into 5 X $10^{•}$ D3 ES cells under the following conditions. Sub-confluent ES cells were trypsinized, washed once in PBS and resuspended in 900• l PBS (5.6 X 10^{7} cells/ml) with DNA. Cells/DNA were placed in a Bio Rad electroporation cuvette and electroporated at 500• F, 0.2KV. Cells were immediately resuspended in 10 ml complete DMEM in the absence of puromycin (Sigma). 0.6-3 x 10^{6} cells were then seeded into gelatinized 10cm diameter tissue culture grade petri dishes in 10 ml fresh DMEM plus LIF in the absence of puromycin. After 18 hours, media was replaced with fresh complete DMEM plus LIF and 1-5 • g/ml puromycin. Cells were selected for over a 10-14 day period. Media was changed during selection every 24 hours. Clonal cell lines were initially amplified in gelatinized 24 well plates (Falcon) amplified and

expression of human c-myc confirmed by Western blot analysis using the anti c-myc monoclonal antibody, 9E10 (Evan et al 1985) on crude whole cell lysates.

Preparation of whole cell protein extracts:

Whole cell extracts were prepared by washing cell pellets in ice cold PBS and resuspending in ice cold lysis buffer (250mM KCl, 50mM Hepes pH 7.9, 0.1mM EDTA, 0.1mM EGTA, 0.4mM NaF, 0.4mM NaVO4, 10% glycerol, 0.1% Tween 20, 0.5mM PMSF, 1mg.ml leupepin, 1mM DTT) at $1.5X10^8$ cells/ml. Protein concentrations of extracts were typically 5mg/ml.

LIF titration assay:

LIF titration assays were set up in gelatinized 24 well trays. 900• l of DMEM LIF was added to each well together with varying amounts of recombinant LIF were added to give final concentrations of 40-0 U/ml (ESGro, AMRAD). 500 cells were added to each well and the medium mixed for even spreading. After 6 days the plates were stained for alkaline phosphatase activity.

Alkaline phosphatase staining:

Alkaline phosphatase was visualized using the diagnostic kit 86-R (Sigma). The kit was used according to the manufacturer's specifications with the following modification; cell layers were fixed in 4.5 mM citric acid, 2.25 mM sodium citrate, 3 mM sodium chloride, 65% methanol and 4% para-

formaldehyde prior to washing and staining.

Results

c-myc drastically reduces spontaneous differentiation of ES cells

Examination of c-myc expressing colonies in comparison to control (vector alone, puromycin-selected colonies) revealed striking differences. First, stably-transfected c-myc ES cell lines were far more uniform in shape and size (ie uniform dome shaped colonies; data not shown). Besides forming spherical, domed-shaped colonies, a noticeable reduction in the number of differentiated cells surrounding the colonies was observed. This is in marked contrast to control ES cells which are prone to spontaneous differentiation (5-10 % colonies on a plate normally exhibit a differentiated phenotype) in addition to the presence of differentiated cells at the periphery of colonies. Less than 1% of c-myc transfected cells exhibited a differentiated phenotype (data not shown). These characteristics were consistent in all ES colonies that expressed the c-myc transgene (12 c-myc and 12 control cell lines were characterized).

c-myc reduces the requirement that ES cells have for LIF

To evaluate the stability of c-myc ES colonies and their ability to differentiate, we compared the ability of these cells to retain pluripotency in the presence of reduced levels of LIF. The logic being that if c-myc was promoting pluripotency and blocking spontaneous differentiation, it may substitute, partially or fully, for LIF's stem cell maintenance function. This possibility was

tested by growing control and c-myc-transfected, puromycin-selected ES cells in the presence of LIF over a 40-0U/ml concentration range for 6 days. The assay was morphology-based and on the ability of ES colonies to retain alkaline phosphatase activity, a marker for pluripotency (see Materials and methods). The % of colonies scoring positive for alkaline phosphatase activity is represented in Figure 13 in comparison to vector (pEF-IRES) alone, puromycin-selected ES colonies. Clearly, c-myc expressing colonies have a significant reduction in their requirement for LIF. Significant decreases in c-myc ES cell pluripotency (as judged by alkaline phosphatase staining), were not seen until the LIF concentrations of 5U/ml and above (>75% alkaline phosphatase positive). Control ES cells generally lost pluripotency over the time course of this experiment at and below 20U/ml. These data clearly show that c-myc expressing ES colonies have a significantly reduced requirement for LIF indicating that c-myc has some stem cell stabilizing function. The experiment shown is typical for more than six c-myc and 6 control ES lines tested in this assay. C-myc expression levels in each pc-Myc.puro-transfected cell line was comparable (data not shown) as were the behaviour of the c-myc cell lines by morphological and AP staining critria.

Differentiation of pluripotent cells is inhibited by c-myc

The ability of c-myc to maintain ES cell pluripotency was characterized in further detail using conditions of cell growth that would normally facilitiate the differentiation of cells, resulting in loss of pluripotency. ES cells were converted into pluripotent EPL cells (see Example 1) and grown on bacterial

petri dishes as aggregate-forming EPL embryoid bodies in the absence of LIF (decribed in Rathjen et al 1999). The differentiation of control (vector minus c-myc insert) versus c-myc transfected cells was assessed by Northern blot analysis over an 5-day time course, using Oct4 (marker of pluripotency) and brachyury (marker for nascent mesoderm) probes. Figure 14 shows that vector control cell lines down-regulate Oct4 mRNA and lose pluripotency after 3 days (after LIF withdrawal) whereas all c-myc cell lines tested maintain high levels of Oct4 expression throughout the experiment, confirming that they retain pluripotency. In control cell lines, brachyury mRNA was markedly up-regulated at day 3, indicating the formation of nascent mesoderm. In c-myc cell lines, the expression of brachyury was typically delayed by at least a day and was not up-regulated to the same extent as in vector alone cell lines. This indicates that differentiation of c-myc expressing cells was incomplete and largely blocked. Differentiation that did occur was delayed significantly. Hence, in the absence of LIF, the differentiation of pluripotent cells is severely compromised by the activity of c-myc. This shows that c-myc has properties that allow for the stabilization maintenance of pluripotent stem cells.

Other parameters were also evaluated with regard to the inability of ES cells ectopically expressing c-myc to differentiate (and hence retain pluripotency), such as retention of high cyclin E expression levels and Cdk2-cyclin E activity. In c-myc expressing cell lines, cyclin E protein remained elevated to similar levels as seen in LIF-maintained ES cells (data not shown). Cdk2-cyclin E associated kinase activity decreased approximately 2-3 fold

over the time course of this experiment but was still present at unusually high levels in comparison to other cell lines characterized to date (data not shown). The cell cycle structure of c-myc expressing cells during the time-course of this experiment remained typical of that seen for ES and other pluripotent cells maintained in a stem cell state, on medium containing optimal concentrations of LIF (data not shown). However, this changed significantly during the experiment in vector alone cells, corresponding with differentiation-associated cell cycle remodelling (see Example 1).

Constitutive c-myc/cyclin E activity in pluripotent cells in vitro could be achieved by several approaches, including generation of cell lines carrying transgenes (as described in this Example). Alternatively proteins could be transduced into cells by approaches which include microinjection, electroporation, use of lipid-based transfection reagents or fusion proteins that include a transduction domain, such as the transduction domain of TAT (Nagahara et al 1998).

Controlled differentiation is central to many of the commercial applications of pluripotent cells. For differentiation of the pluripotent cells with ectopic cell cycle regulatory activities, release from these activities is a major advantage. For this reason the use of protein transduction approach to introduce cell cycle regulatory molecules into the cell has significant advantages, since it allows activity to be delivered and persist for just the time required. Levels of cell cycle regulatory molecules, sufficient for effective activity, can be achieved for the time required. Once the activities have served

their purpose, intracellular levels of the transduced protein are reduced as cells replicate, and further reduction is achieved by normal protein degradation. Where cell cycle regulatory activity is achieved by ectopic gene expression, release could be achieved by use of a c-myc, cyclin E or other appropriate expression constructs that includes Cre-lox recognition sequences. The expression construct would be removed from pluripotent cells by application of the Cre-lox system. Alternatively an inducible promoter could be included in the expression construct. Constitutive c-myc or cyclin E expression is achieved so long as the inducer is included in the culture medium, and when differentiation of the pluripotent cells is required, the inducer would be removed.

Example 5: selection against differentiated prb+ cells using ectopic p16 expression: an approach to select for pluripotent stem cells

A system to allow and promote the maintenance of pluripotent stem cells and select against differentiated cells is attractive for several reasons;

it will eliminate differentiated cells that can destabilise pluripotent stem cells, and thus improve conditions under which pluripotent cells can be maintained

(i) it can be used to functionally select for other types of stem cells with similar properties to ES cells. Such properties would also be expected of other stem cells populations that have been difficult to identify, isolate and maintain in vitro.

Our characteriization of pluripotent ES cells has shown that pRb is unable to associate with at least some of its cellular targets (specifically E2F transcription factors, see Example 2). The basis for this is likely to be due to the precocious activity of Cdks such as Cdk2-cyclin E and or Cdk2-cyclin A. These cells can therefore be considered as having no functional pRb, as it is biochemically inactive. As stated previously (see Example 2), this eliminates the need for Cdk4-cyclin D activities and should hence render these cells insensitive to the Cdk inhibitory molecule, p16 (see Jiang et al 1988 and references therein). This marks what is a fundamental difference between the cell cycle regulation of differentiated cells compared to pluripotent cells. This fundamental difference in cell cycle control mechanisms can be exploited for the selection purpose described above as pluripotent cell that do not utilize Cdk4,6-cyclin D activities will be insensitive to p16, whereas differentiated pRb+ cells will be sensitive (Jiang et al 1998).

Materials and methods:

All methods and materials were as described in previous examples except where stated

p16 expression construct and characterization of p16 cell lines:

A Xho I-Not I fragment from the plasmid pKS.mp16 (gift from C. Sherr) spanning the entire coding region of human p16 was subcloned into the vector pEF-IRES (see Example 4). The resulting construct was linearized with Nde I and transfected into D3 ES cells as described previously (see Example 4). Puromycin resistant colonies (1-5 • g/ml puromycin) were cloned, amplified

and analyzed for p16 expression using an antibody raised against p16 (Santa Cruz sc-1207) by Western blot analysis (data not shown). The functionality of the p16 fusion protein was tested by its ability to bind Cdk4 in whole cell lysates. Typical cell lines quantitatively sequestered all Cdk4/Cdk6, indicating that all Cdk4/6 was inactive (data not shown). Differentiated cells under these conditions would exhibit a G1 arrest (Sherr and Roberts 1999). p16 is not normally expressed at detectable levels in ES cells (data not shown).

Selection against differentiated cells using transduced p16 protein

As another approach to determine that p16 can be used to select against differentiated cells, recombinant TAT-p16 fusion proteins were produced (as described in Nagahara 1998) in bacteria. Typically, recombinant TAT-p16 was purified from 200ml culture of cells grown to an OD_{600} 1.0. Cells were collected by centrifugation and resuspended in 20 ml Buffer Z (8M urea, 100mM NaCl, 20mM Hepes pH 8.0). The cell lysate was sonicated for 15 seconds, three times and centrifuged at 12,000 rpm for 15 minutes at 4°C. The supernatant was adjusted to 20mM imidazole and tumbled for 15 minutes in a 50 ml Falcon tube with 5 ml nickel-affinity resin (Qiagen) for purification of the 6X Histidine-tagged TAT-p16 fusion protein. The lysate and beads were poured into a 10ml Bio Rad EconoColumn and after further equilibration and elution of unbound protein with 50 ml Buffer Z, 20mM imididizole, TAT-p16 protein was eluted with 10 ml Buffer Z, 100mM imidizole (1ml fractions). Fractions containing the TAT-p16 fusion were identified by loading 5• l samples from each fraction on a 12% SDS polyacrylamide running gel,

followed by staining of the gel with Coomassie Blue (data not shown). Fractions containing TAT-p16 were pooled and loaded on a 1ml Mono S column (Pharmacia). After sample loading, the column was equilibrated with 4 column volumes of Buffer A (100mM NaCl, 20mM HEPES pH 7.5). TAT-fusion protein was eluted with 4 column volumes of Buffer A in 1M NaCl and collected in 0.5ml fractions. Finally, the TAT-p16 fusion protein passed through a PD-10 desalting column (Pharmacia) as per the manufacturer's recommendations and eluted in DMEM. This material was added directly to DMEM (+LIF) in varying amounts to give varying concentrations of TAT-LIF. In addition to TAT-p16 (where p16 is in an active form), a TAT-p16mut protein was also produced by the same protocol. The mutant form of p16 contains a single amino acid substitution that eliminates Cdk inhibition because p16mut can not bind Cdk4 or Cdk6 (Koh et al 1995). Hence, TAT-p16mut was used as a control in the experiments described in this example (Figure 15).

Results

The applicability of exploiting p16-selection as a means to select against differentiated cells is shown in Figure 15. Purified TAT-p16 or TAT-p16mut fusion proteins were added to cultures of ES cells, and NIH 3T3 cells grown at sub-confluent densities, 14 hours after plating in fresh media. Cell counts were then performed and plotted as a function of time. Representative plots include untreated cells and treatment with 150• g/ml, 75• g/ml of wild type (TAT-p16) or mutant (TAT-p16mut) fusion protein. Media was replaced every 24 hours, containing TAT-fusion protein where appropriate. The data

shown is a typical experiment where each data point is the mean of two independent cell samples treated under the same conditions.

While the proliferation of ES cells was unaffected by either TAT-p16 or TAT-p16mut, cell division in NIH3T3 cells was severely impaired. Flow cytometry data, used to evaluate cell cycle profiles, indicates that while the profile in ES cells was unaffected by TAT-p16, there was a significant reduction in the fraction of S, G2/M cells when treated with TAT-p16, but not TAT-p16mut (data not shown). This is consistent with cell count assays in Figure 15, showing that TAT-p16, but not TAT-p16mut, causes a G1 block/delay in NIH 3T3 cells. Similar growth inhibition was also seen in MEFs and other cultured fibroblast and epithelial cells lines (data not shown) indicating that this is a general response of differentiated cells to TAT-p16.

These results demonstrate that ectopic elevation of intracellular p16 activity blocks the proliferation of differentiated cells, but not pluripotent cells. Hence, this general approach is an effective method for the selection and maintenance of pluripotent cells in vitro. This approach can also be used to select for pluripotent cells from mixed populations of pluripotent and differentiated cells, and should be applicable for applications described in Example 6.

Example 6: Reprogramming of somatic cells

Technology that allows reversion of differentiated cells to a pluripotent/stem cell state or for the selection of such events, has many

applications that include cell therapy approaches for the treatment of diseases, and the genetic manipulation of animals.

Several approaches have been developed to achieve reprogramming, including nuclear transfer technology. In recent developments adult cells have been used as donors in nuclear transfer (Wilmut et al,1997), and has resulted in the production of cloned animals, albeit with very low efficiency. Reprogramming of bone marrow cells has also occurred following bone marrow transplantation, and cells derived from bone marrow have been identified in several non-haematopoietic tissues (Eglitz & Mezey, 1997; Shi et al, 1998).

Despite improvements in the ability to reprogramme partially differentiated or differentiated cells, there are inadequacies with the current technology that restrict its widespread application for commercial, medical and agricultural benefit. Nuclear transfer, particularly with differentiated cells, is very inefficient, and with all currently available technology it is not possible to identify or maintain cells in transitional states of dedifferentiation. Neither is it possible to direct the redifferentiation of reprogrammed cells in a controlled manner.

Major improvements in reprogramming technology are provided by approaches that involve manipulation of cell cycle regulatory molecules.

One approach involves:

the elevation of cyclin E-Cdk2 activity and/or cyclin A-Cdk2 activity, either directly or by promoting the activities of upstream regulators of cyclin E-Cdk2 activity and cyclin A-Cdk2 activity such as c-myc, in differentiated cells. Elevation of these activities can be achieved by transformation of gene expression constructs, or by transduction of cyclin E, Cdk2 and/or Cyclin A/Cdk2 and/or c-myc polypeptides, by methods known to promote polypeptide entry into cells. These methods include electroporation, lipofection, microinjection and use of fusion proteins comprised of a transduction domain such as included in the HIV peptide TAT (Nagahara et al, 1998).

Cells manipulated in this way may revert to a less differentiated state without further manipulation. Alternatively manipulation in this way may be used to "prime" cells so that they are more responsive to other reprogramming signals, such as the signals that operate during nuclear transfer, where the genetic information from differentiated or partially differentiated cells is reprogrammed by transfer into an enucleated oocyte. Use of "primed" cells as nuclear transfer donors, where cell cycle activities more closely resemble those of dedifferentiated cells, would improve the efficiency of reprogramming. The nuclear transfer embryo formed from such "primed" cells may be allowed to develop, leading to the production of a live animal. Alternatively the "primed" cells could be fused with a cytoplast prepared from a pluripotent cell, thus leading to the production of pluripotent cells with the genetic characteristics of the donor-differentiated cell. Nuclear transfer products using both oocyte cytoplasts or pluripotent cell cytoplasts can be used as a source of

pluripotent cells for in vitro genetic manipulation and/or controlled differentiation.

A second approach involves:

the selection of dedifferentiated cells based on their insensitivity to the Cdk inhibitor, p16 and other members of the INK family of Cdk inhibitors with similar activity . For example, pluripotent cells can be 'captured' from a differentiated cell population by selecting against differentiated cells with p16 fusion proteins, where p16 is linked to a transduction domain, such as the transduction domain of the TAT protein. This approach, could be used in a manner similar to that described in Example 5, in several scenarios including the following. (1) where cells spontaneously dedifferentiate in vitro at low frequency. (2) where cells are reprogrammed in response to inductive environmental signals such as provided by enucleated oocytes, enucleated pluripotent cells, inclusion with pluripotent cells in culture, or in differentiating embryoid bodies.

Due to limitations with the current art, dedifferentiated cells formed either spontaneously or in response to an inductive environment, cannot be recognised or maintained in a dedifferentiated state. Use of the Cdk inhibitor p16, or other members of the INK family of Cdk inhibitors with similar activity, allows such dedifferentiated cells to be captured in vitro. Furthermore the maintenance in an undifferentiated state of cells captured by p16 expression, can be enhanced by expression of other cell cycle regulatory molecules such as cyclin E-Cdk2, cyclin A-Cdk2, and c-myc, that enforce pluripotent cell maintenance and proliferation in vitro (see Example 4).

It will be understood that the invention disclosed and defined in the specification extends to all alternative combinations of two or more of the individual features mentioned or evident from the text or drawings. All of these different combinations constitute various alternative aspects of the invention.

It will be understood that the term "comprises" or its grammatical variants as used herein is equivalent to the term "includes" and is not to be taken as excluding the presence of other elements or features.

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CLAIMS

1. A method of regulating the mitotic and/or physiological activities, and differentiation potential of a pluripotent or multipotent cell, which method includes manipulating the expression and/or activity of a cell cycle regulatory molecule including a regulatory molecule selected from one or more of the groups consisting of a cyclin, a cyclin-dependent protein kinase (Cdk), a Cdk inhibitor, upstream regulators thereof or biochemical targets thereof and/or tumour suppressor protein, and molecules displaying similar activities, in a pluripotent or multipotent cell.

2. A method according to claim 1 wherein the activity of the cell cycle regulatory molecule is manipulated by increasing or decreasing the level of expression of said molecules.

3. A method according to claim 1 wherein the pluripotent cells or multipotent cells are selected from one or more of a group consisting of epiblast cells, embryonic stem (ES) cells, primitive ectoderm-like (EPL) cells, primordial germ cells (PGCs) or embryonic carcinoma (EC) cells.

4. A method according to claim 1 wherein the cell cycle regulatory molecule includes a cyclin selected from one or more of the group consisting of cyclin D, cyclin E and cyclin A or a molecule exhibiting similar activity or a functionally active fragment or analogue thereof.

5. A method according to claim 1 wherein the cell cycle regulatory molecule includes a cyclin-dependent protein kinase (Cdk) selected from one or more of the group consisting of Cdk4, Cdk6 or Cdk2 or a molecule exhibiting similar activity or a functionally active fragment or analogue thereof.

6. A method according to claim 1 wherein the cell cycle regulatory molecule includes a Cdk inhibitor selected from the INK, CIP or KIP families.

7. A method according to claim 6 wherein the Cdk inhibitor is selected from one or more of the group consisting of p27, p57, p16, p15, p18, p19 or p21 or a molecule exhibiting similar activity or a functionally active fragment or analogue thereof.

8. A method according to claim 1 wherein the cell cycle regulatory molecule includes a tumour suppressor protein selected from one or more of the group consisting of retinoblastoma protein (pRb), p107 or p130 or a molecule exhibiting similar activity or a functionally active fragment or analogue thereof.

9. A method according to claim 8 wherein the activity or phosphorylation state of the tumour suppressor protein is manipulated.

10. A method according to claim 1 wherein the upstream regulator is selected from the group consisting of one or more of proto-oncogenes *myc* and *ras* and upstream signalling pathways Raf, MAP Kinase or Rho.

11. A method according to claim 10 wherein the upstream regulator is *myc* or *ras* and the activity of the regulator is increased.

12. A method for identifying pluripotent or pluripotent-related cells which method includes analysing the cell population for pluripotent cell cycle characteristics including one or more of pluripotent-specific cell cycle structure; pluripotent-specific expression of cell cycle regulatory molecules; and phosphorylation status of a tumour-suppressor protein(s).

13. A method according to claim 12, wherein the method includes measuring expression of cell cycle regulatory molecules, including a cyclin(s), a cyclin-dependent protein kinase (Cdk), a Cdk inhibitor, upstream regulators of said molecules or biochemical targets thereof.

14. A method according to claim 13, wherein the pluripotent or

pluripotent-related cells are identified by the presence of one or more of the following characteristics:

a pluripotent-specific cell cycle exhibiting a rapid cycle with short gap phases;

elevated constitutive expression and/or activity of cyclin E;

elevated constitutive expression and/or activity of cyclin A;

pluripotent-specific expression of Cdk inhibitors; and

presence of a phosphorylated tumour suppressor protein.

15. A method according to claim 14 wherein the pluripotent-specific cell cycle structure is characterised by short periods of the cell cycle in G1 and G2/M phases with a reduced G2 phase or absence thereof, with a remodelling of the cell cycle on differentiation.

16. A method according to claim 13 wherein the cyclin E-kinases are constitutively active at levels more than approximately 50 times those for rapidly dividing primary or transformed somatic cells.

17. A method according to claim 14 wherein the Cdk inhibitors p16 and/or p21 and/or p27 are substantially reduced or absent.

18. A method according to claim 14 wherein the method is used to identify other cells derived by partial differentiation of pluripotent cells and/or multipotent cells.

19. A method of facilitating maintenance and/or promoting proliferation of pluripotent cells *in vitro*, said method including

manipulating the expression and/or activity of a cell cycle regulatory molecule, including a regulatory molecule selected from one or more of the groups consisting of a cyclin, a cyclin-dependent protein kinase (Cdk), a Cdk inhibitor, upstream regulators thereof or biochemical targets thereof and/or

tumour suppressor protein, and molecules displaying similar activities, in a pluripotent or multipotent cell such that some or all of the features of the cell cycle of pluripotent cells are enforced.

20. A method according to claim 19 wherein constitutive cyclin E activity and/or constitutive cyclin A activity is increased.

21. A method according to claim 19 wherein constitutive cyclin E activity and/or cyclin A activity is increased by enforcing expression of exogenous cyclin E and/or cyclin A activity, and/or enforcing expression of Cdk 2.

22. A method according to claim 19 wherein the level of constitutive cyclin E activity and/or cyclin A activity is increased utilising the proto-oncogenes *myc* and *ras*.

23. A method according to claim 19 wherein constitutive cyclin D activity is increased by expression of exogenous cyclin D or expression of Cdk 4 and/or Cdk 6.

24. A method according to claim 19 wherein tumour suppressor proteins are inactivated such that maintenance and proliferation of pluripotent cells is prolonged.

25. A method according to claim 24 wherein the tumour suppressor protein pRb is inactivated utilising hyperphosphorylation, antisense techniques or gene inactivation.

26. A method according to claim 19 wherein the upstream regulator is selected from the group consisting of

one or more of proto-oncogenes *myc* and *ras* and upstream signalling pathways Raf, MAP Kinase or Rho.

27. A method according to claim 26 wherein the upstream regulator is myc or ras and the activity of the regulator is increased.

28. A method for reprogramming of differentiated or partially differentiated cells to a less differentiated state which method includes

manipulating the expression and/or activity of a cell cycle regulatory molecule, including a regulatory molecule selected from one or more of the groups consisting of a cyclin, a cyclin-dependent protein kinase (Cdk), a Cdk inhibitor, upstream regulators thereof or biochemical targets thereof and/or tumour suppressor protein, and molecules displaying similar activities, in said differentiated or partially differentiated cells.

29. A method according to claim 28 wherein the differentiated or partially differentiated cells are reprogrammed to a state of pluripotency.

30. A method according to claim 28 wherein the cell is dedifferentiated to a less differentiated or multipotent state.

31. A method according to claim 28 where the cell or nucleus thereof is used as a donor in nuclear transfer.

32. A method according to claim 28 which method includes upregulating cyclin E-Cdk2 and/or cyclin A-Cdk2 activity to direct the differentiated cells or partially differentiated cells towards pluripotency.

33. A method according to claim 28 wherein the upstream regulator is selected from the group consisting of one or more of proto-oncogenes *myc* and *ras* and upstream signalling pathways Raf, MAP Kinase or Rho.

34. A method according to claim 33 wherein the upstream regulator is myc or ras and the activity of the regulator is increased.

35. A method of selecting pluripotent or multipotent cells from a mixed cell population including pluripotent or multipotent cells and differentiated cells which method includes

manipulating the expression and/or activity of a cell cycle regulatory molecule, such that the proliferation and maintenance of differentiated cells are reduced, wherein the regulatory molecule is selected from one or more of the groups consisting of a cyclin, a cyclin-dependent protein kinase (Cdk), a Cdk inhibitor, upstream regulators thereof or biochemical targets thereof and/or tumour suppressor protein, and molecules displaying similar activities, in a pluripotent or multipotent cell.

36. A method according to claim 35 which method includes preferentially selecting for the presence or absence of expression of cell cycle regulatory molecules characteristic of a pluripotent cell cycle including elevated cyclin E-associated activities, elevated cyclin A-associated activities and distinct Cdk inhibitor profiles.

37. A method according to claim 36 which method includes enforcing Cdk inhibitor expression and selecting for survival and maintenance of cells exhibiting elevated levels of cyclin E/Cdk2 activity and/or cyclinA/Cdk2 activity.

38. A method according to claim 37 wherein the Cdk inhibitor is a member of the INK family of Cdk inhibitors, or a molecule with similar activity.

39. A method according to claim 38 wherein the Cdk inhibitor is p16.

40. A method according to claim 37 which method includes introducing into said cells a p16 fusion protein or transformation of the cells with a constitutive p16 expression construct and selection for survival and maintenance of cells.

41. A method according to claim 35 wherein the cells selected are

multipotent cells.

42. A method according to claim 35 wherein the multipotent or pluripotent cells are cells derived by reversion from a differentiated or partially differentiated state to a less differentiated state.

43. A method according to claim 42 wherein the reversion is spontaneous or environmentally induced.

44. A method according to claim 35 wherein the cells are in a transitional pluripotent state.

45. A method according to claim 42 which method includes initiating ectopic proto-oncogene expression and selecting for cells exhibiting survival and maintenance.

46. A method according to claim 45 wherein the proto-oncogene is *myc*.

47. A method according to claim 35 wherein the maintenance and/or proliferation of the selected pluripotent or multipotent cells is facilitated by further manipulating the activity of a cell cycle regulatory molecule.

48. A method of regulating the differentiation of pluripotent or multipotent cells which method includes manipulating the expression and/or activity of a cell cycle regulatory molecule, including a regulatory molecule selected from one or more of the groups consisting of a cyclin, a cyclin-dependent protein kinase (Cdk), a Cdk inhibitor, upstream regulators thereof or biochemical targets thereof and/or tumour suppressor protein, and molecules displaying similar activities, in a pluripotent or multipotent cell, in said cells.

49. A method according to claim 48, wherein the differentiation of the pluripotent or multipotent cells is regulated by one or more of the following

manipulations:

increasing the activity of the tumour suppressor protein;

reducing cyclin E and/or cyclin A-associated activities;

the activity of Cdk2 becomes cell cycle regulated; or

upregulating the activities and/or expression of CdK inhibitors.

50. A method of regulating the cell cycle of primary or untransformed cells, which method includes

manipulating the expression and/or activity of a cell cycle regulatory molecule, such that the proliferation and maintenance of differentiated cells are reduced, wherein the regulatory molecule is selected from one or more of the groups consisting of a cyclin, a cyclin-dependent protein kinase (Cdk), a Cdk inhibitor, upstream regulators thereof or biochemical targets thereof and/or tumour suppressor protein, and molecules displaying similar activities, in a primary or untransformed cell.

51. A method according to claim 50 wherein the lifespan of the differentiated or partially differentiated cells is prolonged by one or more of

upregulation of Cyclin E-, Cyclin D- or Cyclin A-associated activities, or upregulation of upstream regulators of cyclin-associated activities.

52. A method of identifying differentiating cells in a mixed cell population, which method includes analysing the cell population for differentiating cell cycle characteristics including

differentiation-specific cell cycle structure;

differentiation-specific expression and/or

activity of cell cycle regulatory molecules

the presence of active tumour suppressor proteins.

53. A method according to claim 52 wherein the differentiating cells are identified by the presence of one or more of the following characteristics:

a differentiation-specific cell cycle exhibiting a relatively slow cycle with prominent gap phases; and/or

tumour suppressor protein dependency;

reduced constitutive expression activity of cyclin E and/or cyclin A; and

upregulated Cdk inhibitor activity, inlcuding p16, p21 and/or p27, and

Cdk2 activities being cell cycle regulated.

54. Mammalian or avian pluripotent cells whenever produced according to any one of claims 1, 12, 19, 28, 35 and 48.

55. Human pluripotent cells whenever produced according to any one of claims 1, 12, 19, 28, 35 and 48.

56. Pluripotent cells that are competent for genetic manipulation whenever prepared according to any one of claims 1, 12, 19, 28, 35 and 48.

57. Multipotent cells and partially differentiated cells whenever produced according to any one of claims 1, 12, 19, 28, 35 and 48.

58. Human mammalian or avian multipotent cells and partially differentiated cells whenever produced according to any one of claims 1, 12, 19, 28, 35 and 48.

59. Embryos derived from pluripotent stem cells whenever produced according to any one of claims 1, 12, 19, 28, 35 and 48.

60. Animals derived from embryos according to claim 59.

61. Genetically modified animals derived from pluripotent stem cells produced according to any one of claims 1, 12, 19, 28, 35 and 48.

62. Use of pluripotent or multipotent cells whenever produced according to any one of claims 1, 12, 19, 28, 35 and 48, in therapies selected from any one of the group consisting of cell therapy, gene therapy, cancer therapy, regeneration and/or development of organs or appendages or limbs, production and/or use in pharmaceuticals and/or diagnostics, xenotransplantation, genetic modification of animals and nuclear transfer.

63. Use of pluripotent or multipotent cells whenever produced according to any one of claims 1, 12, 19, 28, 35 and 48, in drug delivery.

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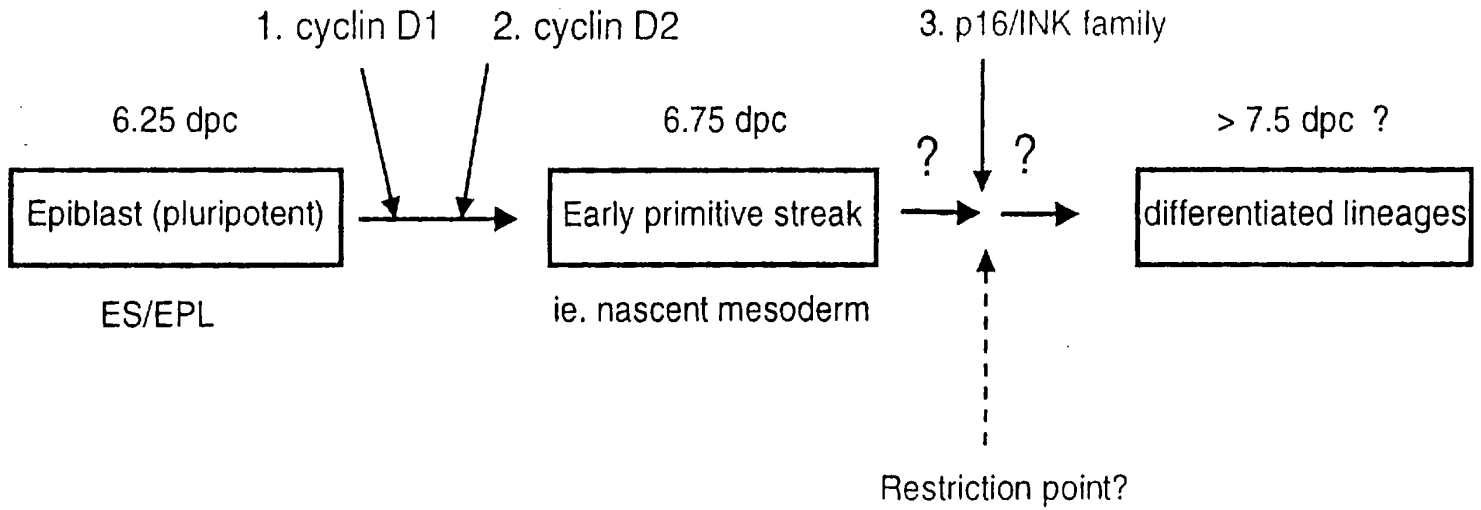
(54) Title: RECOGNITION OF DIFFERENCES IN CELL CYCLE STRUCTURE BETWEEN STEM AND DIFFERENTIATED CELLS

(57) Abstract: The invention resides in the recognition of differences in cell cycle structure between stem and differentiated cells. Pluripotent cells spend ~65 % of time is S phase, ~15 % in G1 and ~20 % in M. Partially differentiated cells have a different cell cycle structure, e.g. mesoderm spend 15-20 % of time in S phase, and 60 % in G1. These differences in cell cycle have lead to the identification of differences in expression of cell cycle genes and activity of regulators. The claims are directed to manipulating cell cycles genes, regulators and proteins to regulate cell activity or cells cycle, identify cell types, facilitate maintenance, reprogramme cells, or regulate differentiation.

mitogenic signals
active Cdk
E2F
E2F
•activation of target genes
•cell proliferation
early G1
R-point (G1)
G1 → S-phase
Restriction (R-) Point Control
Normal somatic cells
1. Regulated activation of pRb-kinases (Cdks)
2. pRb phosphorylation
3. Derepression of genes required for G1-S progression
Embryonic cells (tumour cells)
1. no regulated pRb kinase (Cdk2-cyclinE)
2. pRb constitutitively hyper-phosphorylated
3. E2F target genes constitutively active

Figure 1

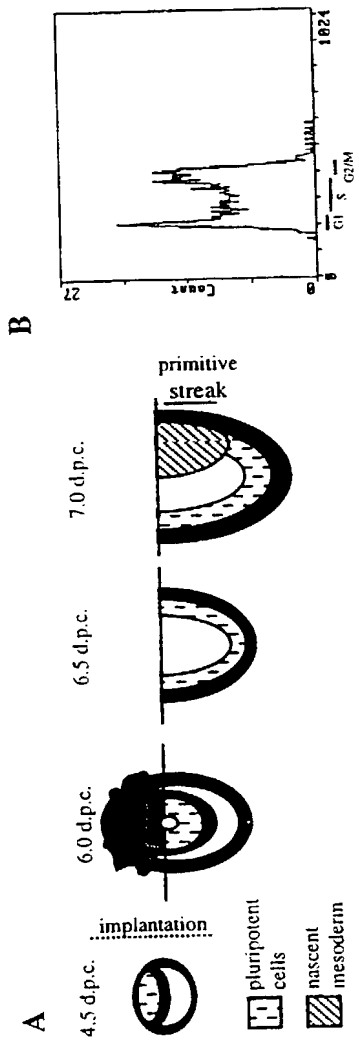
Remodelling of the cell cycle during embryonic development/differentiation



cell cycle length	8 hrs	4-6 hrs rapid proliferative phase	20-24 hrs

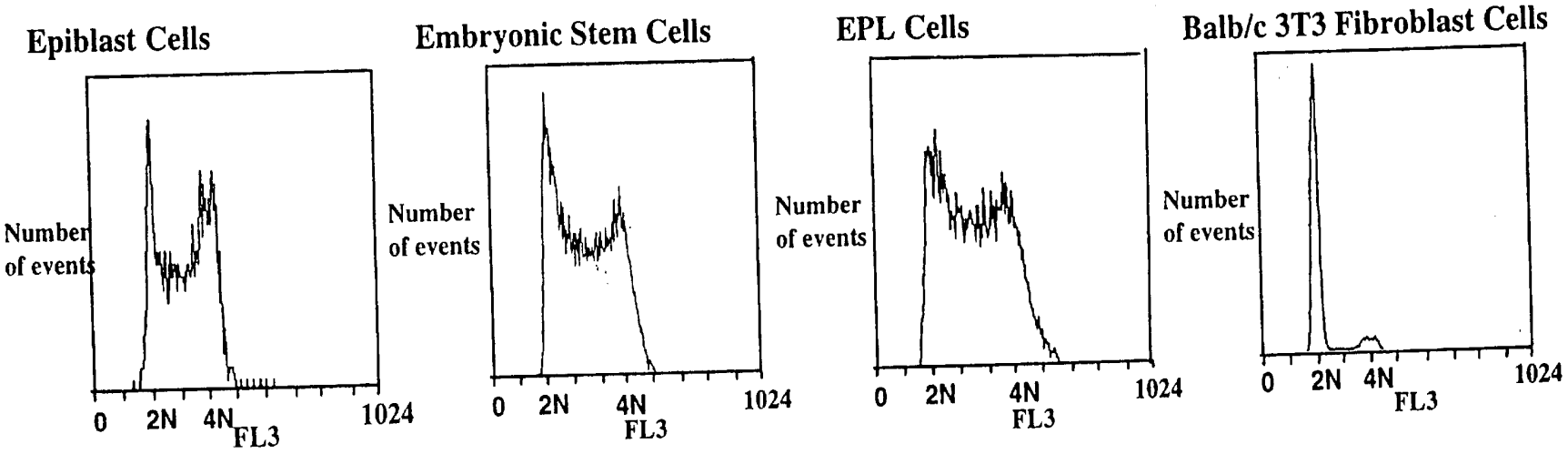
Figure 2

Figure 3

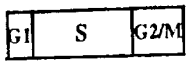


Early mouse embryogenesis. A. Schematic representation of mouse embryogenesis, between 4.5 and 7.0 d.p.c, highlighting the pluripotent cell populations and prior to and during the onset of gastrulation. Gastrulation initiates at the primitive streak and results, initially, in the formation of mesoderm. B. Pluripotent cells from 6.5 d.p.c. embryos were isolated, labeled with propidium iodide and subjected to flow cytometry analysis.

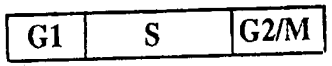
Figure 4



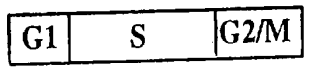
Epiblast Cells (4.4 hours)



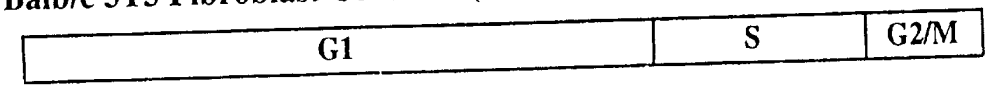
Embryonic Stem Cells (10 hours)



EPL Cells (8-10 hours)



Balb/c 3T3 Fibroblast Cells (24 hours)



PCT/AU00/01184

Figure 5

Cell cycle remodelling during differentiation of cells in embryoid bodies

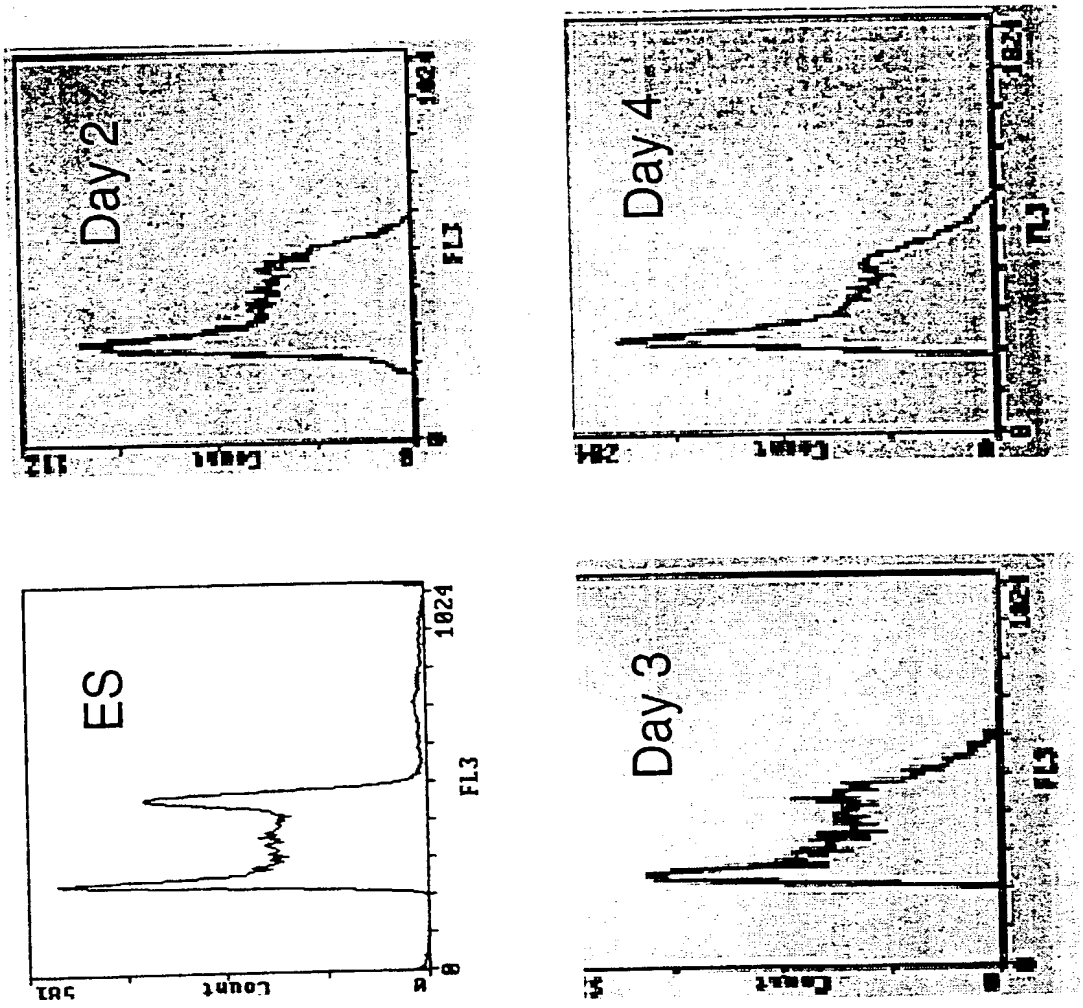


Figure 6

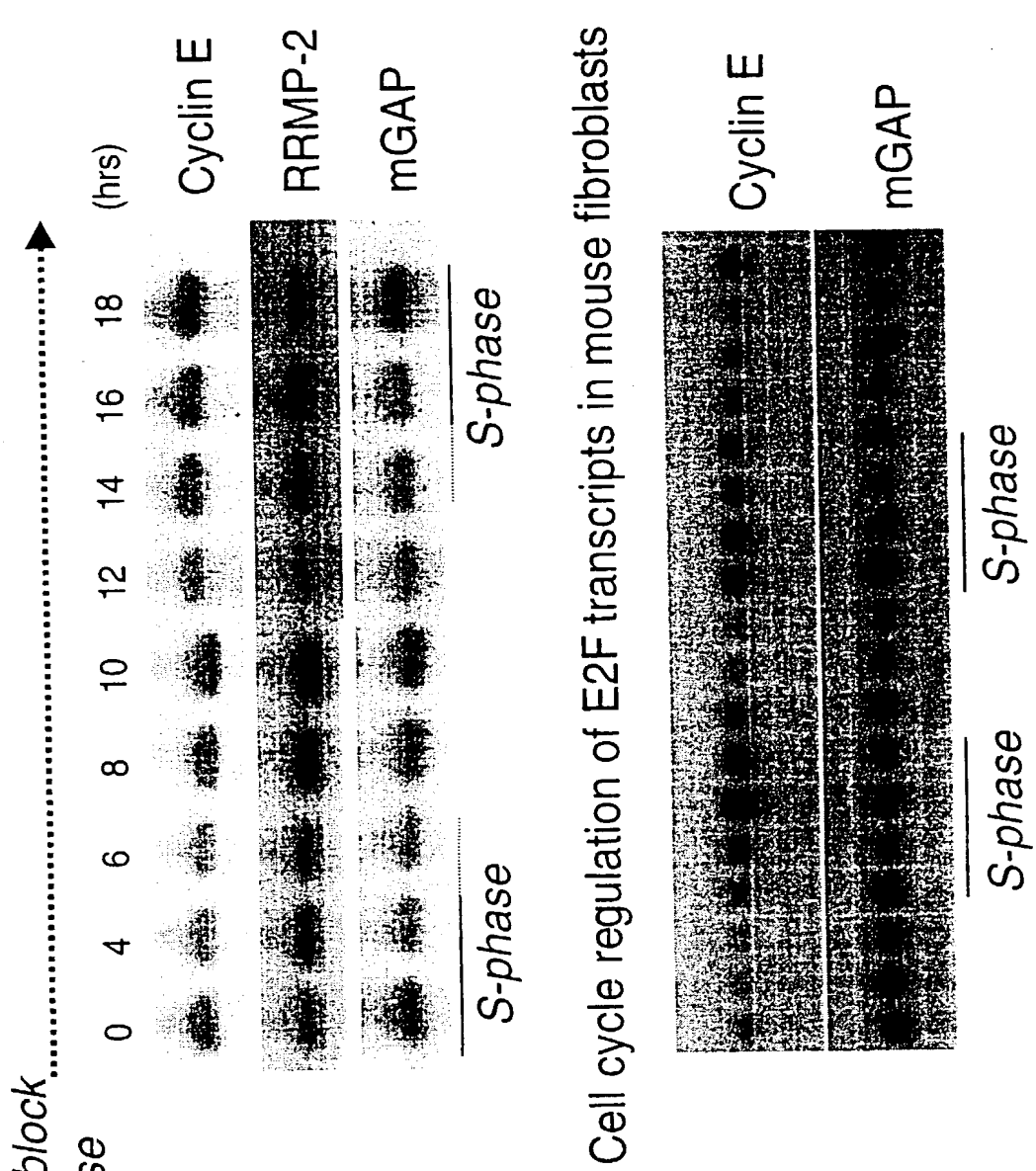
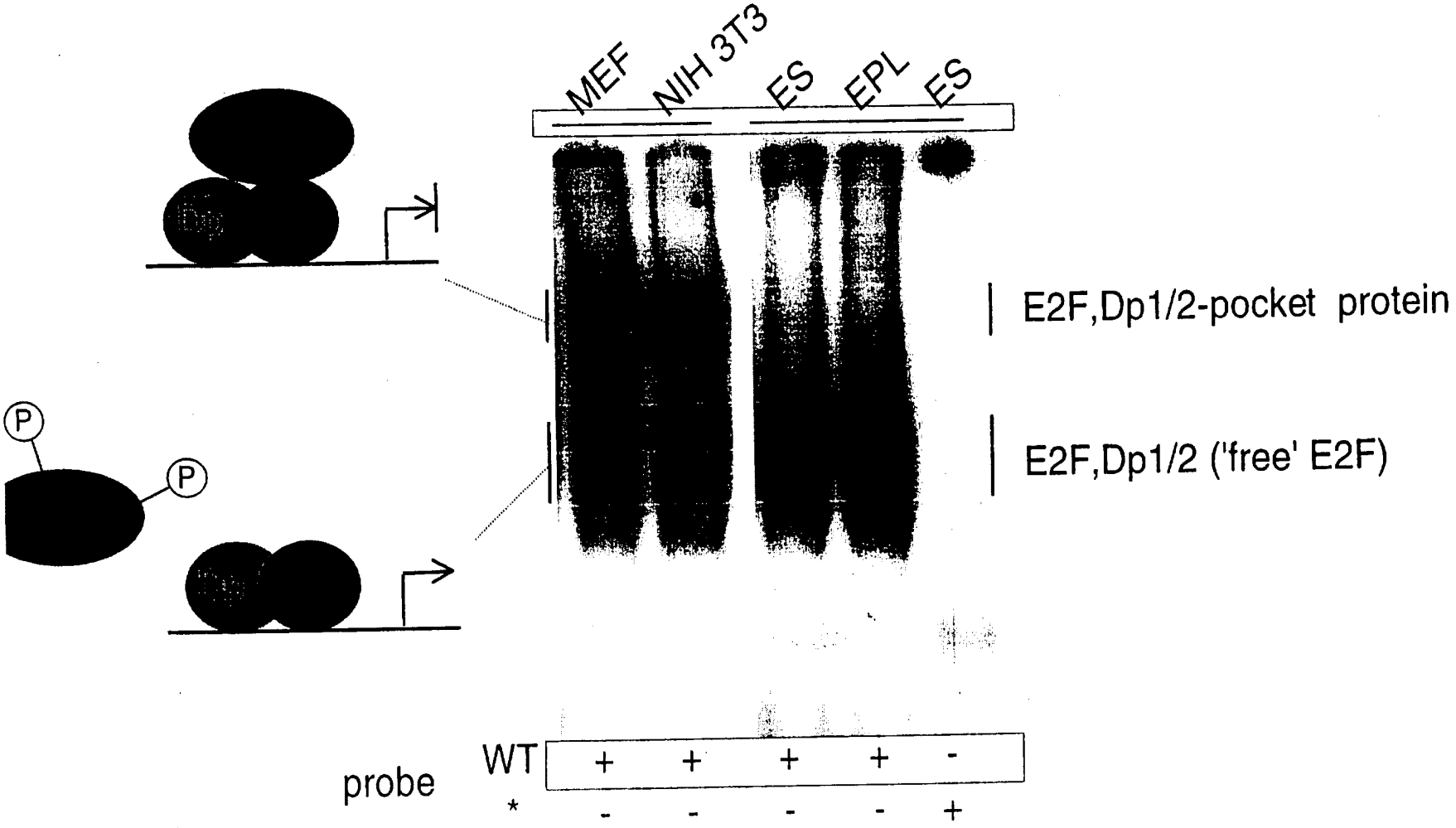

E2F target genes are not cell cycle regulated in ES cells
G1/S-block /release
0 4 6 8 10 12 14 16 18 (hrs)
Cyclin E
RRMP-2
mGAP
S-phase
S-phase
Cell cycle regulation of E2F transcripts in mouse fibroblasts
Cyclin E
mGAP
S-phase
S-phase

Figure 7



E2F complexes in pluripotent cells are predominantly free of pRb family members

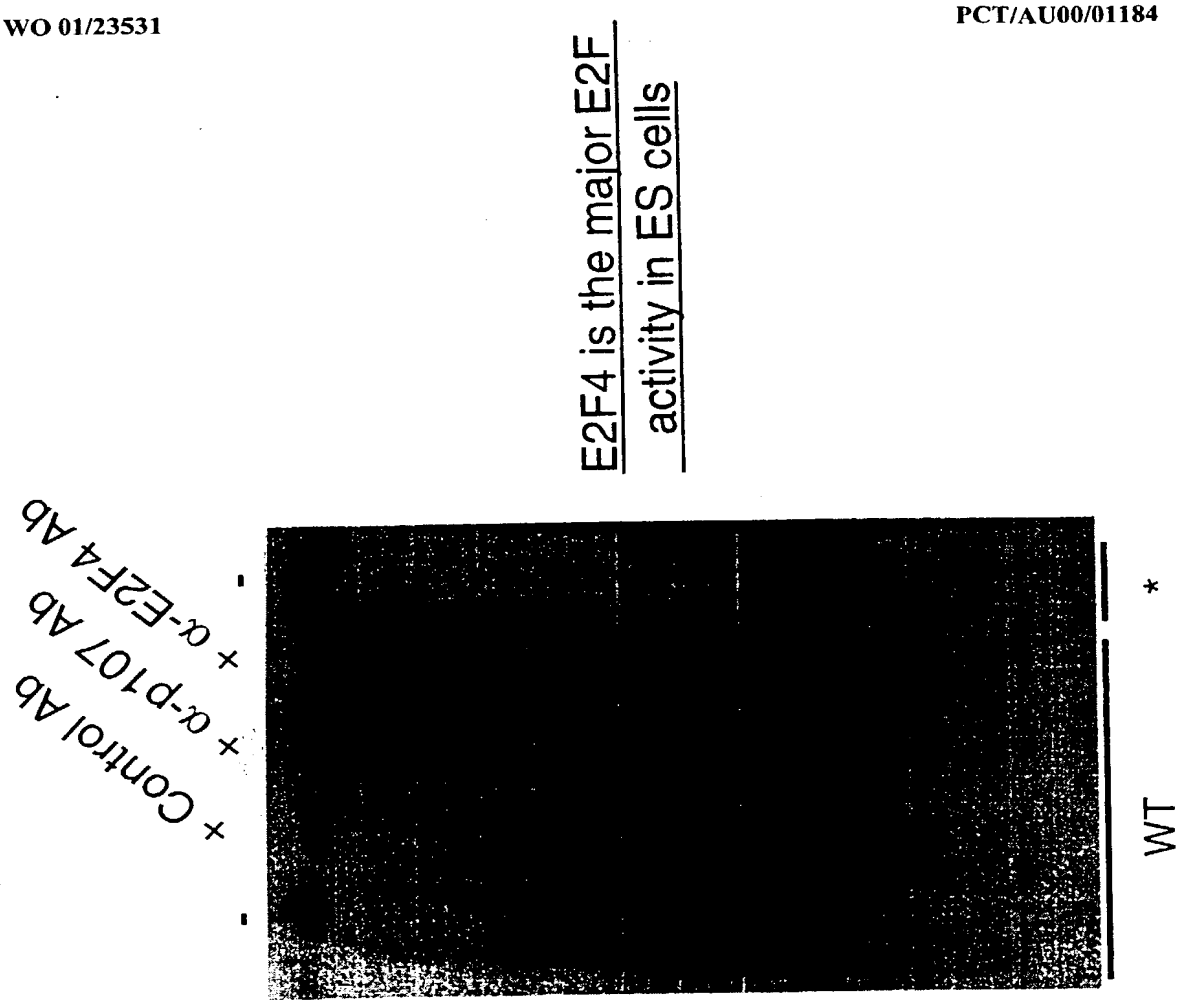

E2F4 is the major E2F activity in ES cells
- + Control Ab
- + α-p107 Ab
- + α-E2F4 Ab
super-shifted complexes
E2F-pocket protein
'free-E2F'
NS
probe
WT
*

Figure 8

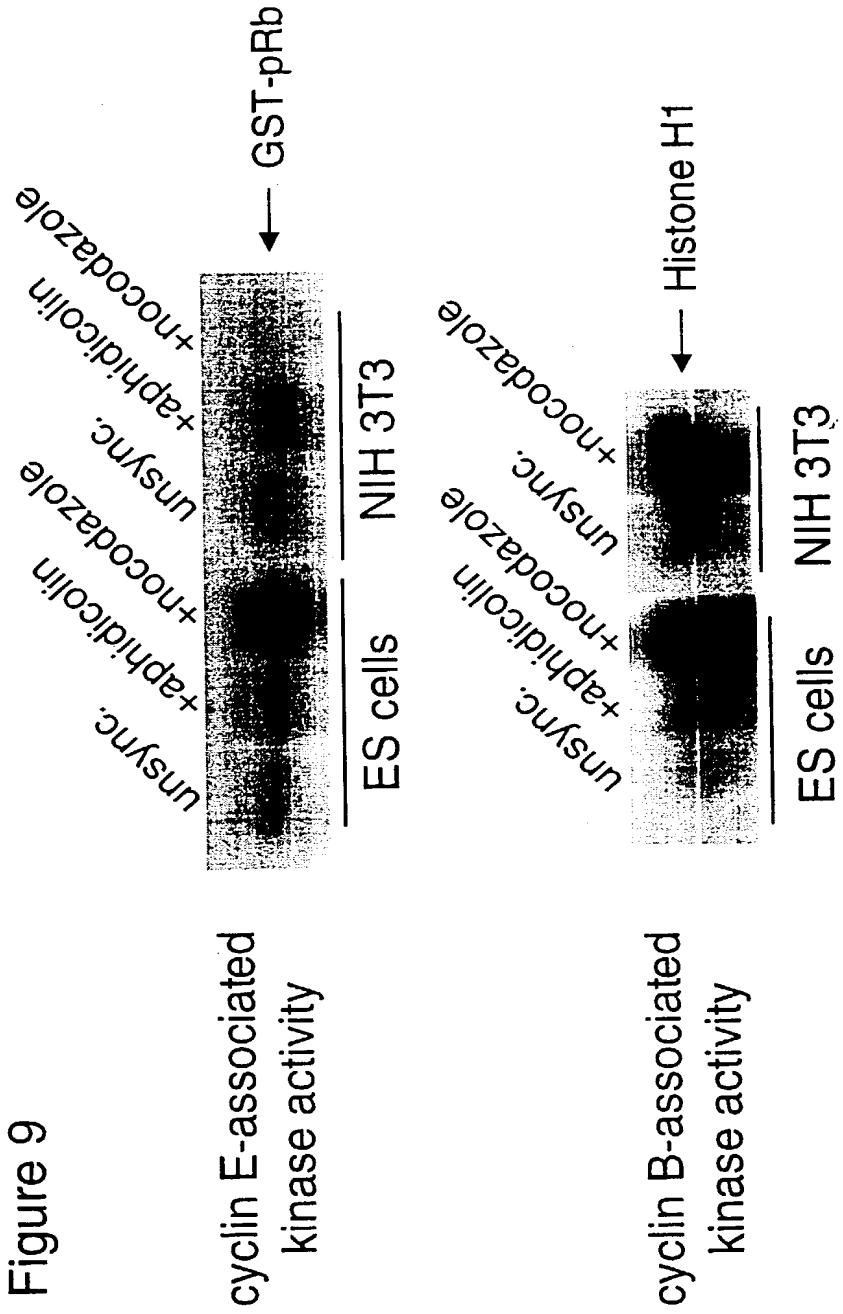

GST-pRb
Histone H1
unsync.
+aphidicolin
+nocodazole
unsync.
+aphidicolin
+nocodazole
ES cells
NIH 3T3
cyclin E-associated kinase activity
cyclin B-associated kinase activity

Figure 9

Figure 10

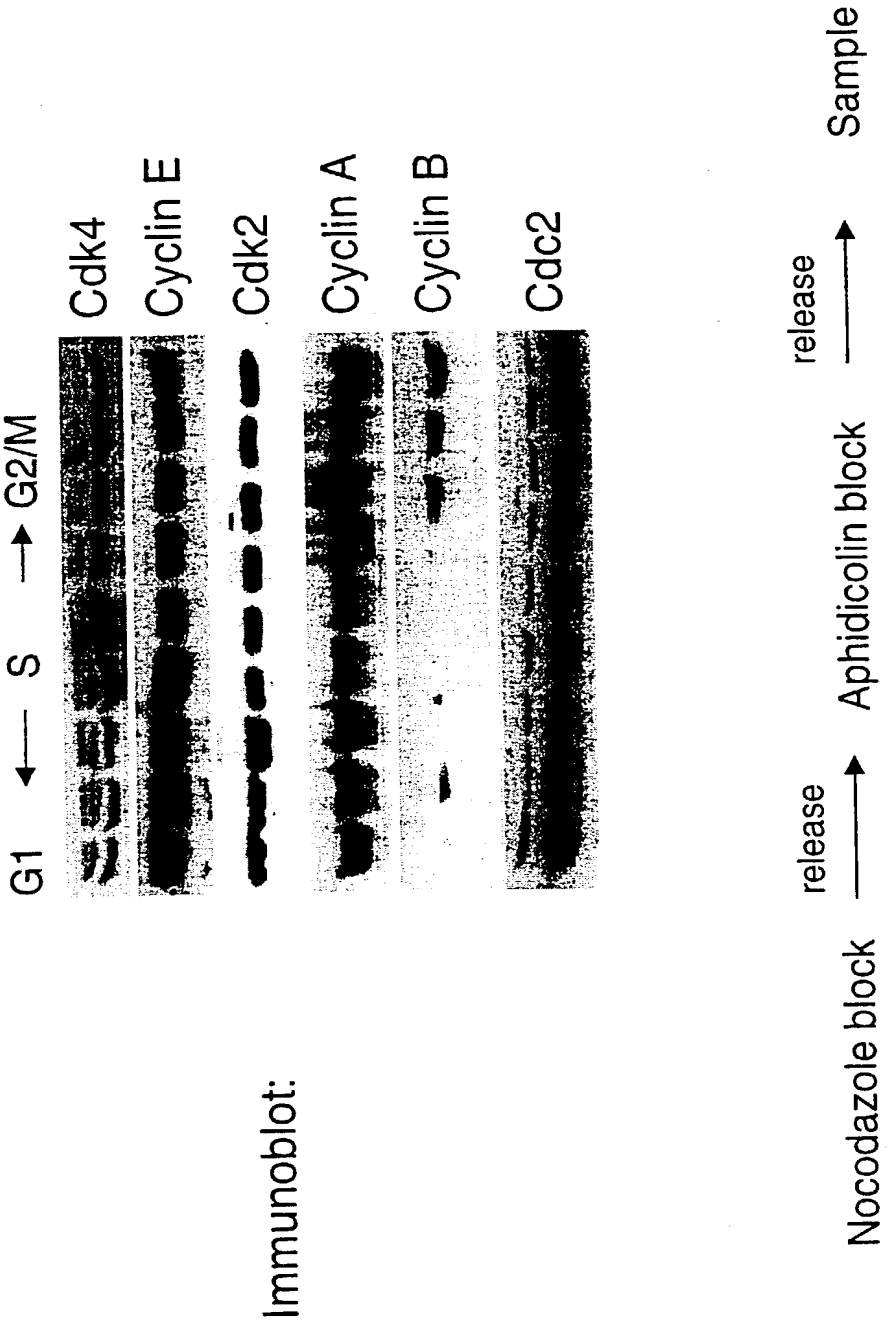
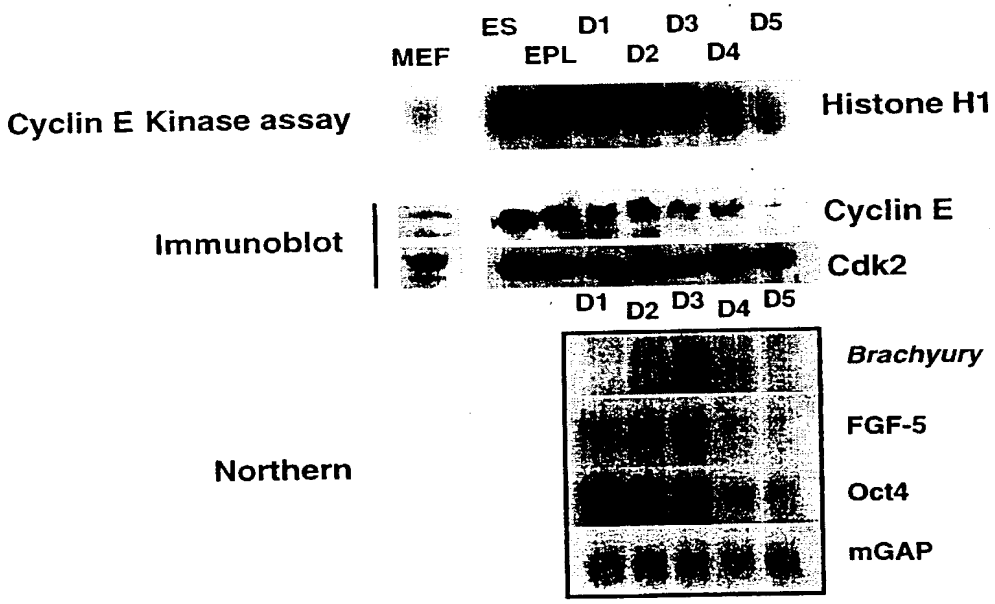

Immunoblot:
G1
S
G2/M
Cdk4
Cyclin E
Cdk2
Cyclin A
Cyclin B
Cdc2
Nocodazole block
release
Aphidicolin block
release
Sample

Figure 11



ES ⟶ EPL ⟶ EPL embryoid bodies ⟶ mesoderm

Cyclin E and cyclin A associated kinase activities collapse during differentiation

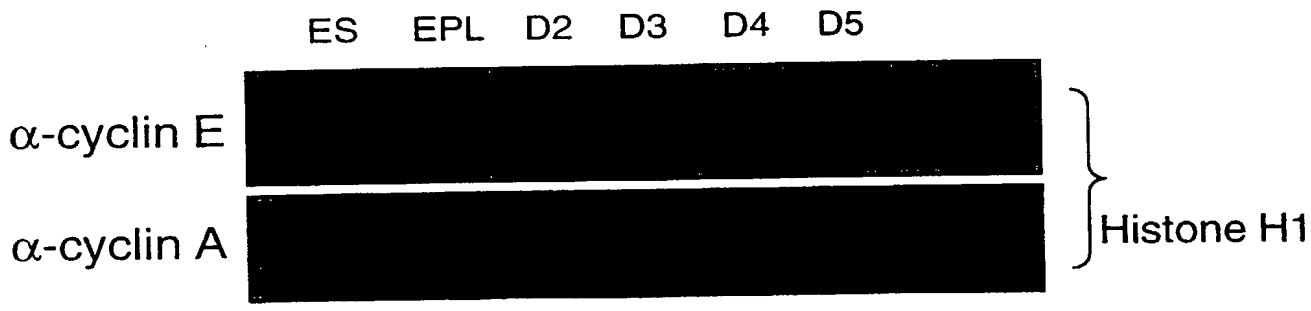


Figure 12

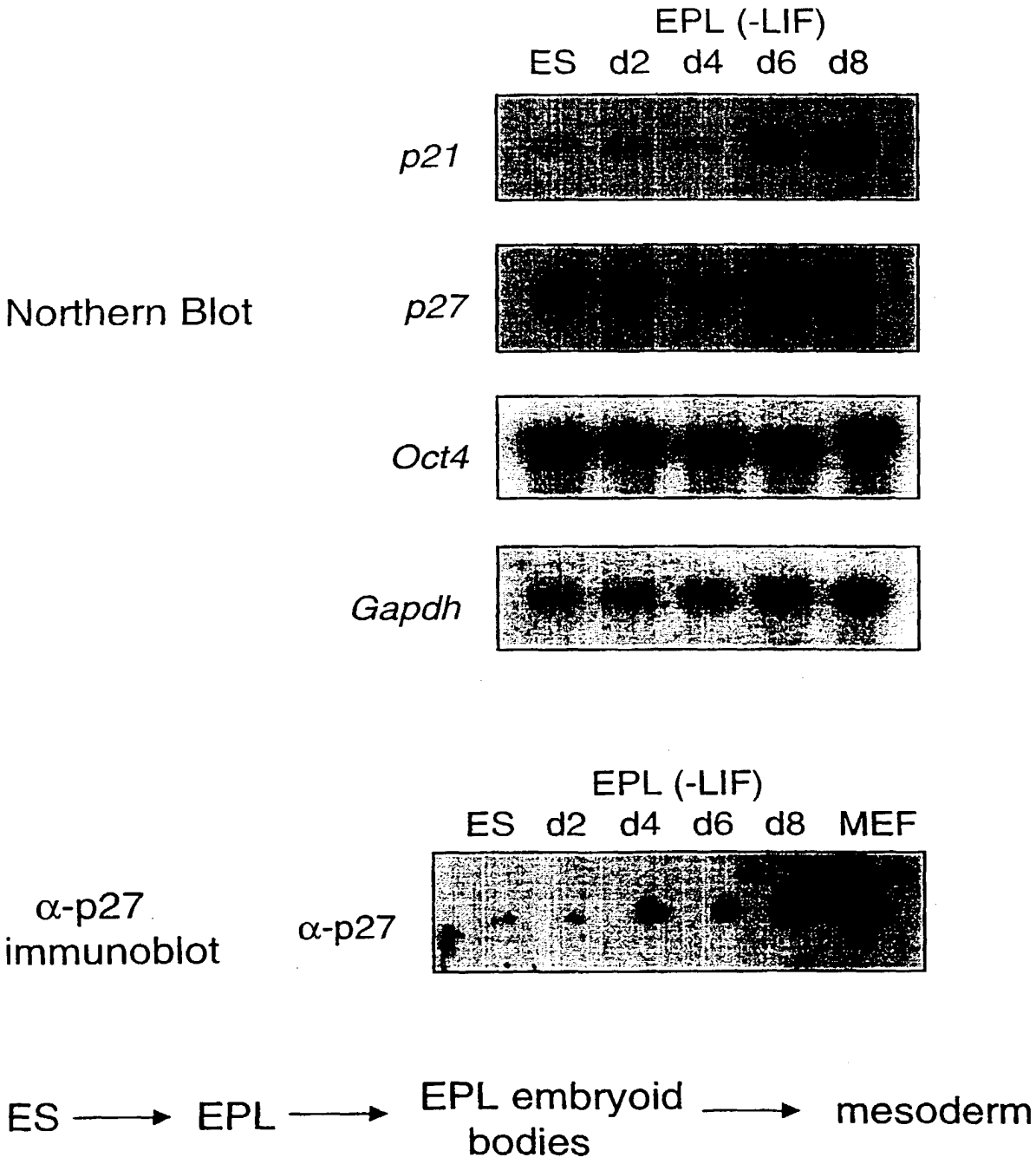

EPL (-LIF)
ES d2 d4 d6 d8
p21
Northern Blot
p27
Oct4
Gapdh
EPL (-LIF)
ES d2 d4 d6 d8 MEF
α-p27
immunoblot
α-p27
ES → EPL → EPL embryoid bodies → mesoderm

Figure 13

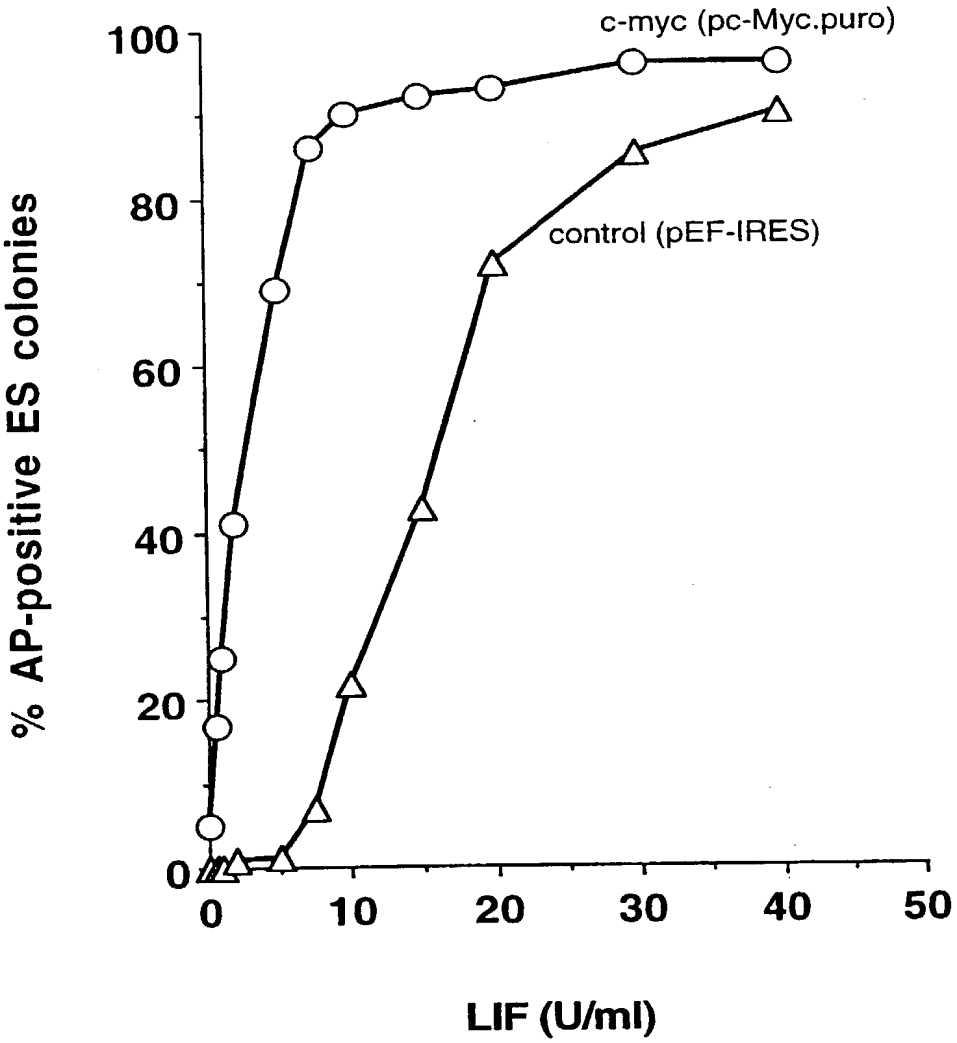
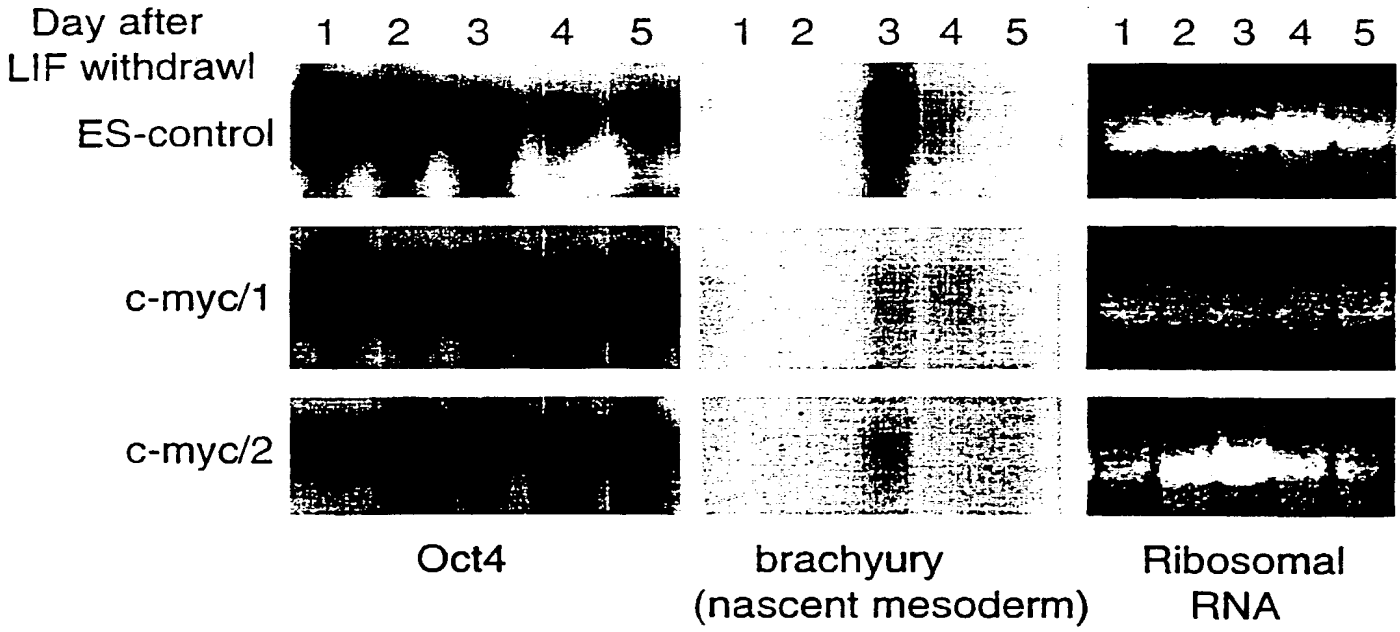
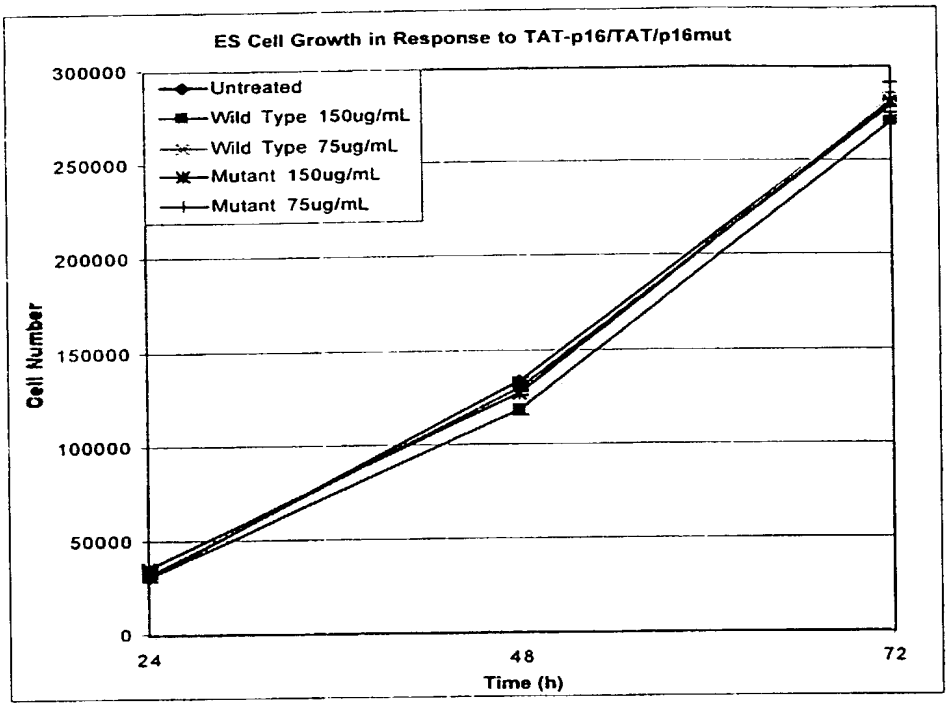


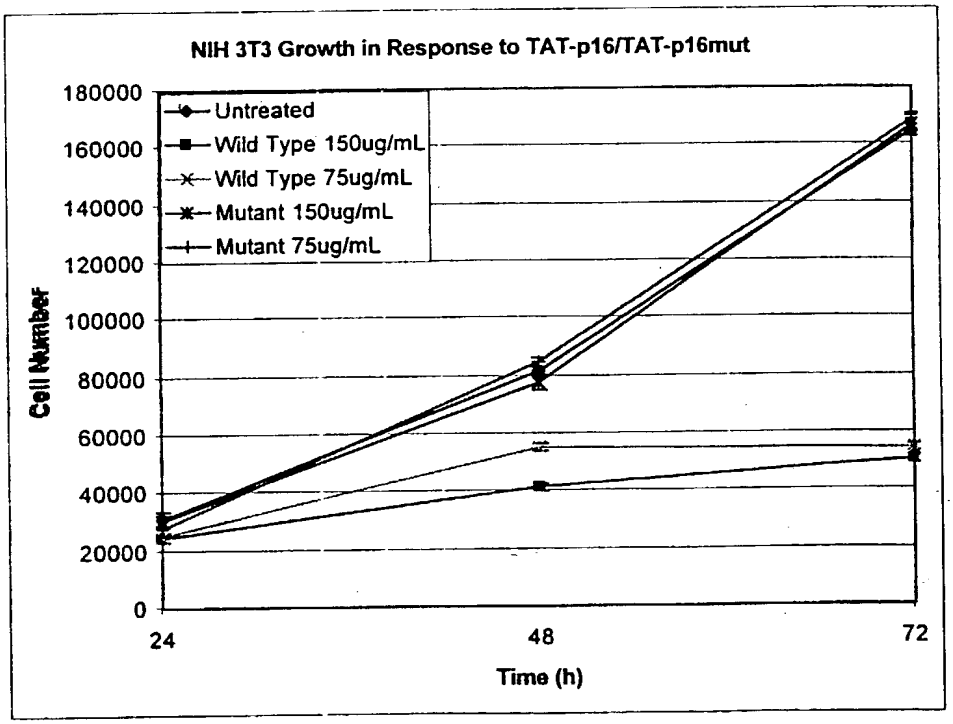
Figure 14



ES → EPL → EPL embryoid bodies → mesoderm

Figure 15


ES Cell Growth in Response to TAT-p16/TAT/p16mut
Untreated
Wild Type 150ug/mL
Wild Type 75ug/mL
Mutant 150ug/mL
Mutant 75ug/mL
Cell Number
300000
250000
200000
150000
100000
50000
0
24
48
72
Time (h)


NIH 3T3 Growth in Response to TAT-p16/TAT-p16mut
Untreated
Wild Type 150ug/mL
Wild Type 75ug/mL
Mutant 150ug/mL
Mutant 75ug/mL
Cell Number
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
24
48
72
Time (h)

Attorney Docket No.:
Title: Recognition of differences in cell cycle structure between stem and differentiated cells
Page 1

(Foreign associate use only)
DECLARATION AND POWER OF ATTORNEY Attorney's Docket No. _____

As a below named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name. I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: Recognition of differences in cell cycle structure between stem and differentiated cells, the specification of which

is attached hereto.

was filed on 22 September 2000 as U.S. Application or PCT International Application No. PCT/AU00/01184 and was amended (if applicable) on _____.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above. I do not know and do not believe that the same was ever known or used by others in the United States of America before my or our invention thereof, or patented or described in any printed publication in any country before my or our invention thereof or more than one year prior to the date of this application. I further state that the invention was not in public use or on sale in the United States of America more than one year prior to the date of this application. *I understand that I have a duty of candor and good faith toward the Patent and Trademark Office*, and I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

to the best of my knowledge

I hereby claim foreign priority benefits under Title 35, United States Code §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below any foreign application for patent or inventor's certificate disclosing subject matter in common with the above-identified specification and having a filing date before that of the application on which priority is claimed:

Country	App. No.	Date of Filing	Priority Claimed Under 35 USC §119
Australia	PQ3073	24 September 1999	Yes X No _____
PCT	PCT/AU00/01184	22 September 2000	Yes X No _____

I hereby claim the benefit under Title 35, United States Code, § 120 of any prior United States application(s), or §365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each claim of the present application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations §1.53, which became available between the filing date of the prior application and the national or PCT international filing date of this application:

Application No.	Filing Date	Status: patented, pending, abandoned

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statement were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patents issuing thereon.

(Foreign associate use only)
DECLARATION AND POWER OF ATTORNEY Attorney's Docket No. _____

As a below named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name. I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: Recognition of differences in cell cycle structure between stem and differentiated cells, the specification of which

is attached hereto.

was filed on 22 September 2000 as U.S. Application or PCT International Application No. PCT/AU00/01184 and was amended (if applicable) on _____.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above. I do not know and do not believe that the same was ever known or used by others in the United States of America before my or our invention thereof, or patented or described in any printed publication in any country before my or our invention thereof or more than one year prior to the date of this application. I further state that the invention was not in public use or on sale in the United States of America more than one year prior to the date of this application. *I understand that I have a duty of candor and good faith toward the Patent and Trademark Office*, and I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

to the best of my knowledge

I hereby claim foreign priority benefits under Title 35, United States Code §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below any foreign application for patent or inventor's certificate disclosing subject matter in common with the above-identified specification and having a filing date before that of the application on which priority is claimed:

Country	App. No.	Date of Filing	Priority Claimed Under 35 USC §119
Australia	[illegible]3	24 September 1999	Yes X No _____
PCT	[illegible]U00/01184	22 September 2000	Yes X No _____

I hereby claim the benefit under Title 35, United States Code, § 120 of any prior United States application(s), §365(c) of any PCT international application designating the United States of America listed below and, insofar as the subject matter of each claim of the present application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations §1.56, which became available between the filing date of the prior application and the national or PCT international filing date of this application:

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I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statement were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patents issuing thereon.

Attorney Docket No.:
Title: Recognition of differences in cell cycle structure between stem and differentiated cells
Page 2

I hereby authorize the U.S. attorneys named herein to accept and follow instructions from _____, as to any action to be taken in the Patent and Trademark Office regarding this application, without direct communication between the U.S. attorney and the undersigned. In the event of a change in the persons from whom instructions may be taken, the U.S. attorney named herein will be notified by the undersigned.

POWER OF ATTORNEY: The following attorneys are hereby appointed to prosecute this application and transact all business in the Patent and Trademark Office connected therewith: Peter G. Pappas - 33,205; Daniel J. Warren - 34,272; William L. Warren - 36,714; Michael S. Pavento - 42,986; Lisa M. Cobern - 44,669; Robert A. Lester - 24,004; Erinn C. Kelly - 44,822; Jerry C. Liu – P47,754; Kevin W. King – 42,737.

Send correspondence to: SUTHERLAND ASBILL & BRENNAN LLP
Direct telephone calls at (404) 853-8000 to _____
999 Peachtree Street, NE
Atlanta, GA 30309-3996

Full name of sole or first inventor: Peter David RATHJEN
Citizenship: Australian
Residence: 1 Mimosa Avenue, South Australia 5051, Australia AUX
Post Office Address: "as above"
Inventor's signature P.D.Rathjen Date: 02-08-02

Peter David Rathjen

Full name of second inventor: Stephen DALTON
Citizenship: Australian
Residence: 43 Carlisle Road, Westbourne Park, South Australia, 5041, Australia
Post Office Address: "as above" AUX
Inventor's signature Date:

Steve Dalton

Attorney Docket No.:
Title: Recognition of differences in cell cycle structure between stem and differentiated cells
Page 2

I hereby authorize the U.S. attorneys named herein to accept and follow instructions from _____ as to any action to be taken in the Patent and Trademark Office regarding this application, without direct communication between the U.S. attorney and the undersigned. In the event of a change in the persons from whom instructions may be taken, the U.S. attorney named herein will be notified by the undersigned.

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Send correspondence to: **SUTHERLAND ASBILL & BRENNAN LLP**
Direct telephone calls at (404) 853-8000 to _____
999 Peachtree Street, NE
Atlanta, GA 30309-3996

Full name of sole or first inventor: Peter David RATHJEN
Citizenship: Australian
Residence: 1 Mimosa Avenue, South Australia 5051, Australia
Post Office Address: "as above"
Inventor's signature P.D.R. Date:

Full name of second inventor: Stephen DALTON
Citizenship: Australian
Residence: 43 Carlisle Road, Westbourne Park, South Australia, 5041, Australia
Post Office Address: "as above"
Inventor's signature Date: 14/8/02

United States Patent & Trademark Office

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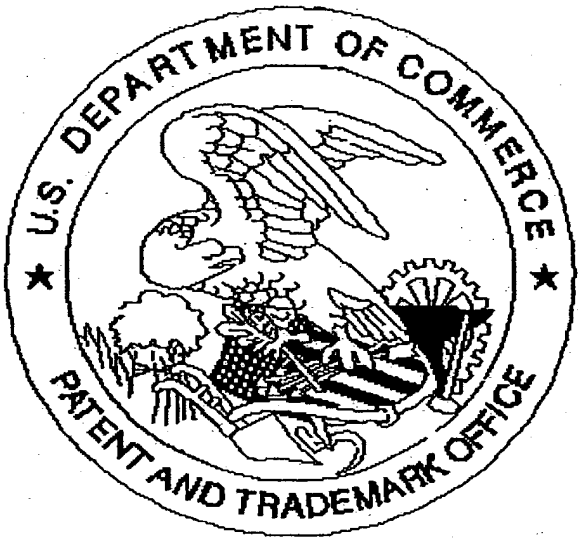

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(Document title)

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